

ESTIMATION OF ADRENAL CORTICOSTEROIDS IN BLOOD.

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CONTENTS.

FOREWORD	page 1.
GENERAL INTRODUCTION	5.

PART 1.

The simultaneous Fluorometric Microdetermination of Cortisol and Corticosterone

1. EXTRACTION	24.
a. Introduction	24.
b. Method	25.
c. Discussion	27.
11. PURIFICATION AND FRACTIONATION OF THE NEUTRAL EXTRACT	31.
a. Adsorption Chromatography	31.
1. Introduction	31.
2. Method	33.
3. Study of the fluorogenic contribution of the various reagents	34.
4. Study of the fluorogenic contribution of silica gel under different condit- ions of activation.....	35.
5. Study of the efficiency of silica gel in the recovery of cortisol	37.
6. Discussion	39.
b. Paper Chromatography	41.
1. Introduction	41.
2. Method	43.
3. Preparation of the paper for chromato- graphy	45.
4. Recovery of cortisol run in the paper	47.
5. Discussion	48.
111. FLUOROMETRY	50.
a. Introduction	50.

	b. Method	page 53.
	c. Determination of the activation and fluorescence wavelengths for cortisol and corticosterone	54.
	d. Specificity	55.
	e. The effect of acid concentration on the fluorescence of cortisol and cortico- sterone	56.
	f. The effect of time on the development of the fluorescence	56.
	g. The effect of temperature on the fluor- escence	57.
	h. Estimation of cortisol and corticosterone in a mixture using two different concent- rations of sulphuric acid	58.
	i. Study of the fluorescence of plasma ex- tract	59.
	j. Elimination of the interfering fluoresc- ence in the plasma utilising the rate of development of the fluorescence	61.
	k. Discussion	64.
IV	FINAL PROCEDURE	68.
	a. The procedure	68.
	b. Evaluation	70.
	c. Advantages	72.

PART 11.

Plasma Cortisol and Corticosterone Levels in the Normal and in some Physiological and Pathological Conditions.

1.	INTRODUCTION	76.
11.	^N NORMAL VALUES	78.
111.	DISCUSSION	78.
IV.	PLASMA CORTISOL AND CORTICOSTERONE IN :	
	a. Pregnancy	81.
	b. Hypo- and hyperadrenal corticism	81.
	c. In response to surgery	82.

SUMMARY	page 86.
ACKNOWLEDGEMENT .	
REFERENCES .	

FOREWORD.

At the present time, when advances in all branches of medicine and biochemistry are rapidly progressing, a great deal of interest is being given to the suprarenal cortex and its functions in health and disease.

Some hundred years ago, the clinical importance of the suprarenal glands was first noted when Thomas Addison, in 1855 described the clinical syndrome which bears his name. He demonstrated the presence of disease of the suprarenal glands in patients with fatigue, hyperpigmentation, hypotension and gastrointestinal irritability (1).

Shortly thereafter in 1856, proof of the vital functions of the adrenals was demonstrated by Brown - Sequard (2) in his classical studies of bilateral adrenalectomy in experimental animals.

Evidence then gradually accumulated about the functions of the adrenal cortex and considerable advances have been made in our knowledge of the elaboration of the adrenocortical hormones. These hormones have been shown to affect directly or indirectly carbohydrate metabolism, electrolyte and water distribution and excretion, sex function and characteristics, and the reaction of the body to infection, cold, trauma and shock.

As early as 1910, Porges (102) pointed out the frequency with which hypoglycemic episodes occurred in patients with Addison's disease and in adrenalectomised dogs. It is now well substantiated experimentally and clinically that it is the C₁₁ oxygenated adrenal cortical steroids that influence carbohydrate metabolism. They do so by stimulating glyconeogenesis from protein (72) and also by inhibiting the peripheral utilisation of glucose (58).

The first observations dealing with inorganic serum constituents were made by Lucas (73) who noted a low chloride level in the blood of adrenalectomised dogs. Gradually, evidence accumulated about the influence of the adrenal cortex on electrolyte and associated fluid excretion and distribution (70,130). Until recently a certain unnecessary confusion prevailed due to failure to distinguish between the effects upon body water and upon electrolytes, and in the case of the latter, to distinguish between the effects on sodium and potassium. It is now established that aldosterone and cortisol, both influence electrolyte and associated fluid distribution, the former being many times more potent than the latter, while cortisol exerts a further important effect on water excretion which is not possessed by aldosterone. Aldosterone and cortisol affect the reabsorption of sodium (and chloride) from the glomerular filtrate as it passes down the renal tubules (138), they are also responsible for the normal cell's ability to maintain ionic gradients and to preserve the essentially extracellular position of sodium. In so far as both the secretion of H^+ and K^+ in the distal renal tubule depends, in part, upon an ion exchange mechanism involving Na^+ reabsorption, aldosterone and cortisol secondarily influence the excretion of these ions. Cortisol in addition, is necessary for the excretion of a water load by the kidney (104).

The adrenal cortex plays an important role in defending the organism against stress and facilitates its adaptation to changes in environment. Selye (118) has summarised the bodily reactions under the term "Stress or Adaptation syndrome", defining three consecutive stages, alarm, resistance and exhaustion. The middle phase is associated with hypertrophy and hyper-

activity of the adrenal cortex and with involution of the lymphoid tissues; the exhaustion phase only occurs if the degree or persistence of shock or trauma exhausts the adrenal defence mechanism.

In the last three decades, chemists began isolating and studying the active principles of the suprarenal cortex. In 1930, the first active extracts of the adrenal glands were prepared, almost simultaneously, by two groups of workers (52,131). The isolation of the active hormones from the adrenal began in 1934 by investigators led by Kendall, Reichstein, and Wintersteiner and Pfiffner. Since then, about 60 steroids have been isolated from the adrenal cortex (16) but only three of them (cortisol, corticosterone and aldosterone) are believed to be the active hormones of the gland (110), the rest being either precursors or metabolites of the natural active hormones.

The next line of attack on the problem of adrenal hormones was the biosynthetic one. Various techniques have been employed in the effort to define the nature of the adrenal cortical secretions. These include an investigation of the crystalline steroid fraction identified in adrenal cortical tissue extracts, chemically and by in vitro studies employing isolated perfused adrenals (54), adrenal slices and homogenates (101,142).

The knowledge from this source has been reinforced by identification of steroids present in peripheral blood and more especially in adrenal vein blood of animals and man, with and without prior stimulation of the gland by corticotrophin (84,23,99).

From all this work, tentative conclusions have been drawn about the nature of the substances actually

secreted by the adrenal cortex. Two steroids of great importance secreted by the gland are cortisol and corticosterone, in various proportions in different species, although the relative importance of the latter is much debated (99). From the physiological standpoint, cortisol and aldosterone are at present best understood.

GENERAL INTRODUCTION.

Since adrenocortical function is deeply involved in the maintenance of biological equilibrium and of life itself, the measurement of the functional states of the adrenal cortices may throw some light on the pathological mechanisms underlying clinical conditions. Their ability to alter their function under a wide variety of environmental stresses is of paramount importance for the clinical biochemist.

The quantitative estimation of the adrenal corticosteroids in urine and in the peripheral blood has proved to be the most valuable method of measurement of the adrenocortical function in man. As a result, it has been possible to come to a better understanding of certain diseases related to adrenocortical dysfunction.

The continued interest in the plasma corticosteroids of man is based in part on the desirability of evaluating adrenocortical function at a level nearer to the source of the hormones, without the interposition of renal factors. In urine the major part of the suprarenal cortical hormones is in the form of conjugated metabolites, but in the blood, almost half of the corticosteroids are present, under normal conditions, as unchanged active hormones (16). This is in part due to the difference between the renal clearance of the free and the conjugated material; the free corticosteroids are cleared from the plasma at a rate of less than one sixth of that of the conjugated material (16). Furthermore, the metabolism of adrenal corticoids appears to be very

rapid; for example, the administration of C^{14} labelled cortisol results in appearance of 15% of the radio-activity in the urine in 1-2 hours and 56% in 6 hours (56). Since conjugation leads to loss of biological activity, from the point of view of the activity of the hormone in the peripheral tissues, a knowledge of the blood level of the free substance is desirable. Estimation of adrenal corticoid hormone activity in the blood will give the hormone level at a definite time, and that level can be followed serially. Blood level of the hormone could also reveal the action of various stimulating or suppressing agents on the secretory pattern of the gland. On the other hand, assessment of the free hormone in the blood will give only a poor indication of the output of the adrenal gland. If that is the aim of measurement, such information could be obtained from urine determination which gives an average daily value reflecting the overall activity of the glands.

The term corticosteroids is usually restricted to the eight known steroids with adrenocortical activity (cortisol, corticosterone, aldosterone, cortisone, cortexone, 11-dehydrocorticosterone, 17-hydroxycortexone, 5-tetrahydrocortisol) and to their inactive metabolites (18). With the exception of cortexone, all have been identified in the peripheral blood of man, some in normal subjects and some in pathological states (87).

Corticosteroids have been measured directly by chemical methods or indirectly by biological methods. A variety of biological assays have been developed utilising the action of cortical hormones

on carbohydrate and protein metabolism of which the estimation of liver glycogen in the fasting adrenalectomised mouse, or the number of eosinophils in the adrenalectomised mouse have been sufficiently sensitive to demonstrate the presence of corticosteroids in plasma. Other bioassay procedures which include the cold protection test in rats, the muscle work test in rats, and thymus weight test in the mouse, were less sensitive and less reproducible. The most sensitive biological method for the detection of adrenocortical hormones was found to be the rat sodium retention test (45).

In 1938, Anderson and Haymaker (3) devised a biological test in which they prolonged the life of adrenalectomised dogs by injecting them with serum from patients with Cushing's syndrome. Adrenalectomised dogs injected with serum from normal individuals acted as controls. In 1943, Vogt(139) used the cold protection test in rats as an assay but was unable to demonstrate the presence of protecting substance in the peripheral blood of dogs.

Due to the extremely low concentration of the hormones in the peripheral blood, the venous effluent of the adrenal gland has been examined. The higher blood level in the adrenal vein permitted a more specific and accurate determination of the steroids present. The presence of adrenocortical hormones in adrenal vein blood was demonstrated by various workers: Vogt, in 1943 (139), used the cold protection test in rats, whereas Paschkins and co-workers in 1950 (93) used the mouse liver glycogen deposition

test, and Simpson, Tait and Bush in 1952 (122) the sodium retention test in rats.

The above results seemed to establish the fact that measurable quantities of adrenal cortical hormones are present in adrenal vein blood, but, that the quantity in peripheral blood is not sufficient to give unequivocal responses by bioassay techniques.

The methods pertaining to the bioassay of adrenocortical hormones in the blood represented the beginning of such assays which subsequently progressed to the more reliable chemical determination. The earliest attempt at a chemical method for the assay of corticosteroids in plasma appears to be that of Hemphill and Reiss in 1947 (57) who measured the corticosteroids by applying to the plasma extract the colorimetric reaction of Talbot et al (133). However, their method of extraction was very crude, as phospholipids were not precipitated (32) and the reaction which they used for this determination was quite unspecific.

One of the earliest chemical methods which is simpler and more reliable than biological testing is that of Corcoran and Page (32). In 1948, they published a method for the determination of corticosteroids in urine and plasma. They extracted heparinised plasma with acetone after it had been precipitated with an alcohol-ether mixture. The dihydroxyacetone of the aqueous phase was oxidised to formaldehyde with periodic acid, and the liberated formaldehyde equivalents were then measured by reaction with chromotropic acid. In this method, only the glycol or α -ketol side chain of the steroids ought to react. This method though indeed quite simple, proved to be unspecific and its

values to be too high, owing to the formation of formaldehyde from phospholipids, traces of which could not be eliminated even by repeated precipitation with acetone, (91). The peripheral blood levels that Corcoran and Page found in males and females ranged from 0.11 - 0.42 mg/ 100 ml. Their values in adrenal-vein blood of dogs ranged from 0.24 - 4.8 mg/ 100 ml.

In 1952, Mader and Buck (75) elaborated on a colormetric reaction for cortisol and related ketol steroids. Since the α -ketol grouping on the steroid molecule (as in cortisone, corticosterone and cortexone) imparts reducing properties, it was felt that substances such as tetrazolium, which on reduction forms red-water-insoluble pigments, known as formazans, would be useful in a colorimetric procedure. Alcoholic solutions of steroids that contain the 1 ry α -ketol group reduce tetrazolium salts in the presence of tetramethylammonium hydroxide and form coloured solution. The authors (75) stated that this reaction was sensitive to 10 μ g/ ml. of cortisone acetate. Cortisone acetate as well as cortexone acetate, corticosterone, 17-hydroxycortexone acetate and 11-dehydrocorticosterone acetate, gives rise to colour with triphenyl tetrazolium and bis-diphenyl tetrazolium.

In 1953, Chen and his associates (30) adopted this reaction to the quantitative estimation, in plasma, of the total free steroids having an α -ketol side chain, utilising blue tetrazolium. They described two methods of performing this colormetric procedure; one using methanolic sodium hydroxide and blue tetrazolium, and the other using ethanol, choline

and blue tetrazolium. The values obtained were essentially in the same range as methods which involved measurements of formaldehydogenic steroids. Apparently the method measures a large group of reducing steroids. Tetrazolium blue is also reduced to some extent, by certain structural features other than an α -ketol side chain(29). Hence the reaction is quite non-specific (31). In order to improve the specificity of the method, a paper chromatographic procedure has been used prior to the development of colour reaction (29). However, this method still gives values more than twice those determined by polarographic or fluorescence technique.

In 1952, Nelson and Samuels (91) published the first practical method adaptable to the routine determination of 17-hydroxycorticosteroids in blood. This method measures mainly cortisol and depends on the presence of 17,21-dihydroxy-20-ketone grouping and is based on a reaction first observed by Porter and Silber in 1950 (103). Corticosteroids, such as cortisol, having this side chain, give a yellow colour with maximal absorption at 410 m μ when treated with phenylhydrazine in sulphuric acid. Nelson and Samuels (91) chromatographed the plasma extract on columns of magnesium silicate-celite or Florosil, to separate corticosteroids from other substances. They obtained 3 eluent fractions; the 17-hydroxycorticosteroids were in the third fraction. These workers also used Allen's correction equation (2) by measuring the absorption light on both sides of 410 m μ to correct for the interfering chromogens. The authors also stated that the colour reaction obeyed Beer's law. Recoveries of 1-4 μ g cortisol added to 10 ml. of plasma and run through the Florosil column varied from 83 - 112%. The compound in the third eluate was identified as cortisol by paper chromatography. With this

method, normal blood level ranges from 4 - 10 μg /100 ml. While column chromatography renders the method somewhat more specific, it probably makes it less suitable for routine assays. In a critical evaluation of the method, Harwood and Mason (53) characterised it as "a difficult but remarkably reliable tool".

In 1953, Bondy and Altrock (55) published a method for the analysis of 17-hydroxycorticosteroids in plasma. It involved a complex extraction of plasma through several steps. The final extract was obtained in a chloroform-carbon tetrachloride mixture which was then evaporated to dryness and the Porter-Silber colour reaction applied. From 60 - 70 % of hydrocortisone acetate was recovered when this steroid was added to plasma. There appear to be many steps in the extraction where steroid recovery could be diminished. Their normal values were $7.8 \pm 3.3 \mu\text{g}/100 \text{ ml}$. Low values were found in hypopituitary patients, Addisonian patients and adrenalectomised patients, and the plasma level increased after corticotrophin administration. Studies on adrenal-vein blood revealed that the concentration of Porter-Silber chromogens was higher than in peripheral blood. The rate of secretion of adrenal hormone was estimated by simultaneously collecting blood samples from the left renal vein and left renal artery and determining the difference in hormone concentration. The difference was multiplied by the value for the renal plasma flow. The calculation revealed a secretion of 15-21 mg./day which was stated to be approximately equal to the amount necessary to maintain patients with adrenal insufficiency.

In the same year, Bayliss and Steinbeck (58) modified the method of Nelson and Samuels slightly. This involved a modification of Porter-Silber colour reaction, namely running blanks which contained the solvents alone and also

known amounts of cortisol along with the unknowns. They made their correction in this manner instead of applying Allen's formula, as did Nelson and Samuels. When cortisol was added to plasma recoveries were 68-93%. They attempted to characterise the steroids found in peripheral blood by paper chromatography and found that from a third to a half of the colour developed was due to cortisol, the remainder being due to more polar compounds. These findings differed from those of Nelson and Samuels.

Kassenaar and co-workers (58) extracted plasma with chloroform and put the extract through a series of refining steps, the residue was then subjected to a modified Porter-Silber colour reaction. The normal plasma level ranged from $6.1 - 6.3 \mu\text{g}/100 \text{ ml}$ - lower than with most methods. In Cushing's syndrome, the plasma level was $20 \mu\text{g}/100 \text{ ml}$., and after administration of ACTH to normal subjects, values were up to $38 \mu\text{g}/100 \text{ ml}$.

In 1954, Silber and Porter (121) used their reaction (103) to develop a method for the determination of 17, 21 dihydroxy 20-ketosteroids in human plasma. This method differed from that of Nelson and Samuels in that column chromatography was not employed. This omission has greatly simplified the technique, and yet normal values determined by these two methods were in very good agreement. Their method involved chloroform extraction of plasma to which cortisol ($5 \mu\text{g}$) was added to allow for any loss of steroid in the extraction procedure. An attempt was made to minimise loss of steroid. Thus the alcoholic phenyl hydrazine sulphuric acid reagent was added directly to chloroform then the steroid was extracted from the chloroform into the reagent. The optical density was measured at 410 m μ . The average plasma level in humans was $13.3 \mu\text{g}/100 \text{ ml}$. Compounds B and A and cortexone reacted with Porter-Silber reagent, but

the absorption peak was at 340 - 360 mμ. The method is stated by Gold (47) to be a good one and to be readily adaptable to routine and research use, but not quite as accurate as that of Nelson and Samuels. Though the normal values determined by the two methods are in good agreement; under abnormal or pathological conditions, a difference in the values of plasma corticosteroids as determined by the two methods might be expected (31).

In 1957, Peterson et al (95) devised a greatly simplified Porter-Silber method for the specific determination of cortisol in plasma. After extraction of the plasma with methylene chloride, the extract was transferred into the phenyl hydrazine sulphuric acid reagent and spectrophotometry of the chromogen then directly carried out. This very simple method involves no fractionation, and yet is specific for cortisol. Under the conditions used cortisol is virtually the sole chromogen present, a fact which has been proved by the isotope dilution technique. The complete elimination of side reaction with accompanying substance cannot, however, be guaranteed, and the method should be used with caution (87).

In 1953, Morris and Williams (84) developed a method for the polarographic estimation of individual steroid hormones in the peripheral blood. 50 ml of heparinized blood were extracted with ethyl acetate after the precipitation of proteins by ethanol. Fat was removed by freezing the extract to -10°C and by washing with carbon tetrachloride. A second fat removal was carried out using cilane-treated Hyflo-Supercel column (reversed phase partition). The extracted corticosteroids were then separated by partition chromatography on Hyflo with ethylene glycol-light petroleum-toluene-dichloroethane by the gradient elution technique; testing of the fractions being done with

blue tetrazolium and U.V. absorption. The individual steroid fractions were then converted into the hydrazones of Girard's reagents, and polarography was carried out on the separated fractions for quantitative measurement and identification. When compounds A,B,E,F and S were added to blood, recoveries were better than 80 %.

By means of this procedure 11-dehydrocorticosterone, corticosterone, cortisone and cortisol were identified and estimated in normal human plasma. In normal plasma, they found the concentration of 11-dehydrocorticosterone to be 3.6 ug, corticosterone 7.8 ug, cortisone 3.9 ug and cortisol 8.4 ug per 100 ml. In pregnancy the values were elevated, mostly in the corticosterone and cortisol fractions. The values of all fractions rose after the administration of corticotrophin; but the greatest rises were in the cortisol fraction. Morris and Williams also pointed out that the 17-hydroxycorticosteroids occur mainly in the plasma rather than in whole blood. While the method appears to be quite specific for each of the compounds determined, some of the findings, such as the presence of cortisone (2.5 - 5.0 ug/100ml) in normal plasma, has not been substantiated by other independent analytical methods. Furthermore, the method appears tiresome and delicate to perform, and the purification, especially of the corticosterone fraction does not appear to be adequate, it might be contaminated by a Δ^4 -3 ketosteroid which is not easily separable by chromatography and so far has not been identified (27,87). The high values of cortisone have been attributed to an oxidation of some cortisol during the process of purification (Samuels 1954, cited by 46).

Weichselbaum and Margraf (141) felt that most of the available methods for the determination of plasma cortico-

steroids measured only the 17-hydroxylated corticosteroids. They therefore combined the Porter-Silber procedure (which measures the 17-hydroxylated corticosteroids) with the Mader-Buck blue tetrazolium procedure (which measures the 17-hydroxycorticosteroids as well as the 17-deoxycorticosteroids) and thus measured the 17-deoxycorticosteroid fraction by subtracting the amount of Porter-Silber chromogens from the amount of Mader-Buck chromogen. They extracted the steroids from blood with ethyl acetate, after further refining, they divided the extract into two fractions. One fraction was chromatographed on Florosil for the Porter-Silber reaction and the other fraction was chromatographed on silica (using formamide as the stationary phase) for the Mader-Buck blue tetrazolium reduction reaction. Plasma was also hydrolysed with bacterial glucuronidase in order to obtain the hydrolysed glucuronides apart from the free form. The average value obtained for the plasma level of free 17-hydroxylated corticosteroids in human subjects was 9.5 ug/ 100 ml.; of conjugated 17-hydroxylated corticosteroids 16.8 ug/ 100 ml.; and of the free 17-deoxycorticosteroids, 8.9 ug/100 ml.. Their recoveries averaged 86% for cortisol by the Porter-Silber reaction and 88% for corticosterone by the Mader-Buck, Porter-Silber colour reactions.

Sweat, in 1954 (127) elaborated on a procedure involving sulphuric acid-induced fluorescence of corticosteroids. Wintersteiner and Pfiffner (143) and later Reichstein and Shoppee (106) observed that with concentrated sulphuric acid, certain corticosteroids gave an orange solution which exhibited a green fluorescence when viewed against dark background. Sweat (127) has developed a method based on this phenomenon for the determination of 11-hydroxycorticosteroids in plasma. Five steroids of the pregnane series were found to produce intense fluorescence in ethanolic

sulphuric acid solution. These steroids were hydrocortisone, epihydrocortisone, corticosterone, epicortosterone and Δ^4 -pregnane-11 β ,17 α ,20 β -21-tetrol-3-one. From these studies, the author made the following generalisation:

1. The presence of an hydroxyl grouping at C₁₁ seemed to be necessary for increased fluorescence.
2. The presence of unsaturated ketone in ring A also seemed to exert a minor influence on fluorescence.

In 1954, Sweat introduced a silica gel microcolumn method for the chromatographic resolution of corticosteroids (128). In 1955, he published a method for the measurement of adrenocorticosteroids in the peripheral and adrenal venous blood which depended upon this fluorometric procedure (129). He extracted the blood with chloroform and partitioned the extract between 70% ethanol and petroleum ether and then chromatographed it on silica gel column. The effluent fractions from the column were then quantitatively analysed for corticosterone and cortisol using both the phenylhydrazine reaction and the fluorescence. Human peripheral blood apparently contained cortisol and corticosterone and additional paper chromatographic studies also revealed what seemed to be compounds S, A and E. Sweat (129) observed that the average concentration of cortisol in normal peripheral blood was 10.8 ug /100 ml. whereas that of corticosterone-like compound was 4.3 ug/100 ml. When corticotrophin was given to normal subjects there was a two-to-five-fold increase in cortisol and corticosterone in peripheral blood, but when corticotrophin was administered to Addisonian patients there was no significant increase in the level of plasma steroids.

In 1958, Silber and ~~Saunders~~ workers (120) working with an almost crude rat plasma reported a method in which they

found that chromatography can be omitted and that fairly specific fluorometry for corticosteroids was possible. Several workers (51,81) confirmed the simplicity and reliability of this method.

The tracer technique proved an important advance in the assay of corticosteroids. The losses of the steroids which inevitably occur during purification can be calculated by the addition of known amount of steroid labelled with a radioactive isotope, the activity of which can be independently measured.

In 1956, Bojesen (10) devised a method for the determination of cortisol in peripheral plasma with radioactive p-iodophenyl-sulphonic acid anhydride (pipsan). The principle depended on esterifying the steroid to be measured with S³⁵-labelled pipsan, the amount of activity present in the ester would be a measure of the unknown amount of steroid present, provided the esterification of that steroid was quantitative, a fact which had been proved. A known amount of radioactive steroid ester labelled with another isotope (I¹³¹) was added. The activity of the latter was independently measured and calculation made to correct for the losses. This is an example of the double tracer technique. This method is highly specific, sensitive and accurate (87).

Bondy et al (11) added 4C¹⁴ cortisol to chloroform plasma extract. After paper chromatography the cortisol zone was eluted and the radioactivity and the soda fluorescence of the eluate were measured. The calculation was then performed of the corrected cortisol values by allowing for losses and added cortisol.

In the same year, Peterson and co-workers (95)

published three papers utilising the isotope dilution technique for the assay of cortisol and corticosterone in plasma and biological fluids. For cortisol (95), the method was very similar to that of Bondy et al (11) except that methylene chloride was used for extraction of plasma and the cortisol zone eluate was measured for its radioactivity and for the Porter-Silber chromogen. For corticosterone (94), carbon tetrachloride-methylene chloride 1:1 was used to extract the plasma to which 4-C^{14} -corticosterone had been added, this ensured that no steroids more highly polar than corticosterone were removed. Paper chromatography was then carried out in a solvent system of cyclo-hexane-benzene-methanol-water (4:4:2:1). The corticosterone zone eluted and determined for its radioactivity and sulphuric acid fluorescence. Using a double tracer technique for steroids in biological extracts, Peterson and co-workers (96) acetylated the extract with H^3 -labelled acetic anhydride and mixed it with C^{14} -labelled acetate of each steroid to be determined. Intensive fractionation was then performed by chromatography; losses did not matter since they could be calculated. The H^3 - and C^{14} - activity of the separated substances were then measured and the steroid content calculated.

These types of determination offer the maximum sensitivity, specificity and accuracy, provided that the chemical reactions involved proceed quantitatively. However, the problem of extraction and fractionation still remained.

In addition to the freely extractable fraction, the metabolites and a small amount of the hormones are present as glucuronides which can be split without difficulty with β - glucuronidase. Various investigators have studied

the conjugated corticosteroids in the plasma. Bongiovanni and associates (17) have found that a good part of the circulating blood corticoids is in the conjugated form.

Southcott and co-workers (123) have shown that when corticotrophin was administered, there were rises in the levels of both the free and conjugated fractions.

Table (1) summarises most of the methods which are related to the assay of the free adrenocortical hormones in the blood.

It will be evident that the chemical determination of plasma corticosteroids invariably involves, as the first step, extraction of the plasma usually with an organic solvent. After this initial extraction, most methods attempt at some means of purification, a notable exception being the methods described by Silber and Porter (121), Peterson and co-workers (95), Silber et al (120), De Moor et al (33) and Moncloa et al (81), who merely relied on the selectivity of the extracting solvent and the specificity of the reaction used for determination.

Other methods depended on partitioning the extract between an aqueous ethanol or methanol and petroleum or toluene or hexane. (12,59,106,140).

Some workers (91,129) employed a relatively simple chromatographic procedure. This has made their methods time-consuming and more difficult, although by such means many non-specific substances were removed from the extracts. On the other hand, many workers have insisted on a thorough purification and separation of the corticosteroids before quantitative determination. Some used paper chromatography after column prechromatography (27) or two stage paper chromatography (11). This separation and purification of

the corticosteroids, has increased the specificity and the accuracy of the method but has rendered it still more difficult and more time consuming, and a large amount of blood was required, rendering the method less suitable for routine clinical investigations.

After these steps, a number of reactions can be used for determination of the corticosteroids. Since it has been demonstrated that cortisol is the predominate corticosteroid of the normal peripheral blood in man (27), it is obvious that any chemical method useful for the determination of corticosteroids of human plasma must measure the level of cortisol.

Several different methods are available for quantitative chemical determination of steroids possessing the following structures:

1. Δ^4 -3 keto group by ultraviolet spectrophotometry (109)
2. Δ^4 -3 ketosteroids show a specific yellow fluorescence in aqueous sodium hydroxide in U.V. light 360 mu. (21).
3. α ketol group and the glycol group, after oxidation with periodate give formaldehyde (52).
4. The 17,21 dihydroxy-20-ketosteroids give a yellow colour when treated with phenyl hydrazine sulphuric acid reagent (103), (Porter-Silber reaction).
5. All steroids with α ketol or dihydroxyacetone side chain have reducing properties and can be oxidised by blue tetrazolium or other tetrazolium salts. (75)
6. Polarography for determination of steroids with reactive ketogroup as Girard derivatives (84).
7. Sulphuric acid or phosphoric acid fluorescence for a number of steroids especially those possessing hydroxyl groups (143).
8. Tracers methods either with one (95) or two different

isotopes (95).

The term "corticosteroids" has been loosely applied to those steroid fractions which were determined by various methods. It would, however, be more informative if the term were modified to give rise to such terms as: "Formaldehyde-hydrogenic corticosteroids" or "Reducing corticosteroids" or "Porter-Silber chromogen" (7). However, if measurements are made with methods which are only relatively specific, more specific terms might be used.

From the above review of the common methods used for the assay of plasma corticosteroids, it is evident that these methods can broadly be subdivided into two groups: those analytical methods which try to perform a very thorough Laboratory investigation and those which aim at a simple practical clinical routine method.

Only during the last few years has it been possible to develop specific methods from the existing, relatively un-specific methods for the determination of the individual corticosteroids. It is now necessary to develop, refine and perfect these methods so that they can become reliable methods for clinical and research use.

The main aim in this thesis is to develop a method which, on the one hand, should be accurate and specific, and on the other hand to be as simple and time-saving as possible.

The majority of the techniques described by different authors utilise the Porter-Silber reaction in the final determination of the corticosteroids. This reaction is group specific and can only detect the 17-hydroxycorticosteroids namely cortisol, cortisone and 17-hydroxycortexone (cpd S) but it fails to detect 17-deoxycorticosteroids;

corticosterone and 11-dehydrocorticosterone (cpd A). Most authors believe that cortisol constitutes the major part of the free plasma corticoids in normal persons. Morris and Williams (84) and Weichelbaum and Margraf (141), however, insist that the concentration of corticosterone is about the same as cortisol. The fact that the Porter-Silber reaction does measure small amounts of plasma corticosteroids other than cortisol even in normal persons and the possibility that under abnormal conditions, it might determine predominantly an entirely different corticosteroid should be recognised. (31). The Porter-Silber reaction moreover, is not specific for the dihydroxyacetone configuration of cortisol and of related substances. Many carbonyl compounds, including various drugs (119) yield a colour with this reaction.

For determination of corticosterone simultaneously with cortisol, an additional reaction to Porter-Silber is necessary, for example Mader-Buck blue tetrazolium reduction, where the difference between the figures given by these two reactions corresponds to 17-deoxycorticoids. A single reaction which is common to both cortisol and corticosterone and which can simultaneously determine them is their fluorescence in sulphuric or phosphoric acid.

There is much to recommend the fluorometric determination of adrenocortical steroids. It is highly sensitive: in blood the lower limit of sensitivity for cortisol is 0.5 ug for the phenyl hydrazine method (91) and 0.05 ug for the Fluorescence method (129) and therefore it can be adapted as a micro-method. The fluorescence technique is also quite simple. Methods utilising the Porter-Silber reaction have always been complicated by a high blank

value and though several attempts have been made to reduce the blank value, it has not been reduced sufficiently to permit confidence in the absolute values obtained (77). Quenching effects which are commonly observed in the fluorometric analysis of estrogens in urine extracts have not been observed in the fluorometric technique for corticosteroids in blood. (129).

To develop an easy and simple method, intending particularly for application in humans the minimum amount of plasma which can give reliable results is to be preferred. The fluorometric technique seemed to satisfy the required aims and was therefore chosen as the method for the determination of cortisol and corticosterone in blood.

TABLE (1)

METHODS OF ESTIMATION OF ADRENOCORTICAL STEROIDS IN THE BLOOD.

AUTHORS	Plasma Volume	METHOD			No. of Cases	Specificity	Normal $\mu\text{g}\%$ Avr Range		Recovery	Group Reaction
		Extraction	Purification & Separation	Quantitative Determination						
Vogt 1943	50	Acetone	.Protein precipitation by alcohol ether .Removal of fat by petroleum ether .Phospholipid precipit. by MgCl_2	Cold protection test in rats		Formaldehy- -dogenic Steroids	0.11 -0.42 mg.			Biological assay
Paschis 1950				Mouse liver glycogen deposition test						Biological assay
Corcoran & Page 1948				Oxidation by periodic acid to formaldehyde assayed by chromotropic acid						α -ketol group
Nelson & Samuels 1952	10	Ether/ Chloroform 4/1	Chromatography on magnesium silicate- celite or Florosil	Porter-Silber reaction		17-OH-CS	4-10	83-112 % 80-132 %		17,21 dihydroxy- -20-ketone
Gornall & MacDonald 1952	10	Chloroform	Partition between benzene & water	2,4 dinitrophenyl hydrazine						Δ^3 ketone, 17,21 dihydroxy-20- ketone
Morris & Williams 1953	20	Ethyl acetate	.Protein precipitation by ethanol .CCl ₄ wash .Defatting with silane Hyflo-Supercel parti- -tion column .Hyflo partition chro- -matography	Polarography of Girard hydrazones of Δ^3 ketost.	7	Cortisol Corticosterone Cortisone 11-dehydro- -corticosterone	8.5 5-12.5 8.0 4-10.5 4.0 2.5-5 4.0 2-7.5	71 - 92%		
Bush & Sandberg 1953	135 - 300	Ethyl acetate	.Na ₂ CO ₃ wash .Silica gel prechroma- -tography .Paper chromatography	U.V. absorption NaOH fluorescence	6	Cortisol Corticosterone	15 \pm 7 3 3 \pm 2	80 - 100 %		Semiquantitative
Bliss, Sandberg, Nelson & Eik-Nes 1953		Chloroform	Florosil column chromatography	Porter-Silber	91 M 29 F	17-OH-CS	13 \pm 6 15 \pm 6			17,21 dihydroxy- -20-ketone
Bayliss & Steinbeck 1953	15	Chloroform	Florosil column chromatography	Porter-Silber	91	17-OH-CS	9.5 \pm 3.3 3 - 16	68-93 %		17,21 dihydroxy- -20-ketone
Bondy & Altrock 1953	15-20	Isopropyl alcohol	.Protein precipitation by Methanol/Ether 3/1 .Complex purification in several steps by solvent partition	Porter-Silber	30	17-OH-CS	7.8 \pm 3.3 3 - 13	60-70 %		17,21 dihydroxy- -20-ketone
Silber & Porter 1954	10	Chloroform	—	Porter-Silber	16	17-OH-CS	13.3 \pm 6.2 6 - 25	95 %		17,21 dihydroxy- -20-ketone

Bongiovanni & Eberlein 1954		.Chloroform .β glucuro- -nidase hydrolysis	Chromatography on Florisil Rechromatography on silica gel	Porter-Silber Acid fluorescence	17-OH-CS	8 ± 1	2-10		17,21 dihydroxy- -20-ketone	
Sweat 1955	5	Chloroform	.Partitioning between 70% ethanol & petroleum ether .Chromatography on silica gel	Sulphuric acid fluorescence	21	Cortisol 10.8 ± 2.6 Corticosterone- like cpds. 4.3 ± 2.3			11-hydroxysteroid	
Weichselbaum & Margraf 1955	20	.Ethyl acetate .β glucuro- -nidase hydrolysis	Column chromatography: .Florisil .Silica gel	Porter-Silber BTZ	29 M 42 F 17 (coj)	17-OH-CS 9.5 ± 1.5 17-deoxy-CS 8.9 ± 2 Conjugates 16.8 ± 1.6		80-90(86) 83-100(88) %	17,21 dihydroxy- -20-ketone & α-ketol group	
Kassenaar et al 1955	20	Chloroform	Partitioning between 70% methanol & toluene-Ligroine pentane	Porter-Silber	21 M 16 F	17-OH-CS 6.8 ± 1.4 6.2 ± 1.5	5-9.5 4.7-8.2	80-104(93) %	17,21 dihydroxy- -20-ketone	
Bongiovanni & Eberlein 1955		.Methylene chloride .β glucuronidase hydrolysis	Florisil chromatography	Porter-Silber	30	17-OH-CS 8.5			17,21 dihydroxy- -20-ketone	
Klein & Papadatos 1955		.Chloroform .Acid hydrolysis	Florisil chromatography	Porter-Silber	30 Adults 45 Child.	Free 17-OH-CS 13 ± 1.9(SEM) Conjugates Adults 8.5 Child. 4			17,21 dihydroxy- -20-ketone	
Chen et al 1955	10-15	Methylene chloride	Paper chromatography	BTZ	54	Free reducing CS	M 35.1 ± 7.9 F 32.4 ± 5.4	22-52 22-43	over 100 %	α-ketol group
Wallace et al. 1955	10	Chloroform	—	Porter-Silber	50	17-OH-CS 16 ± 6.9	4-32		17,21 dihydroxy- -20-ketone	
Bojesen 1956	5-10	Chloroform	Partitioning between methanol and toluene in n-hexane	Double tracer technique	12	Cortisol		70-104%		
Peterson & Wynngaarden 1956	20-30	Methylene chloride	Paper chromatography	.Sulphuric acid fluorescence .Radioactivity	13	Cortisol 14 ± 4			11-hydroxysteroid	
Southcott & Gandossi 1956	175	.Chloroform .β glucuronidase hydrolysis	Paper chromatography	.UV absorption .BTZ .Porter-Silber	12	Cortisol Corticosterone		54.6% 52.3%	4-3-ketone, α-ketol group, 17,21 dihydroxy- -20-ketone	
Lewis 1957	5	Ethyl acetate	Paper chromatography	Sulphuric acid fluorescence	30	Cortisol 9.2 ± 1.5 Corticosterone 1.2	6-12 0-6	92% 87%	11-hydroxysteroid	
Takeda 1956		Chloroform	Silica gel chromatography	Phosphoric acid fluorescence	17	F-like subs. 8.6 B-like subs. 3.4			11-hydroxysteroid	
Bondy & Upton 1957	20	Chloroform	Two-stage paper chromatography	.Sulph. acid fl. .Butoxide fluor. .Isotope dilution for recovery correc.	29	Cortisol 10.2 ± 3.6 Corticosterone 1.3 ± 2.5	4-17.7 0-5	100% 90.9%	11-hydroxysteroid 4-3-ketosteroids Δ	

Scheuer & Bondy 1957	30	Chloroform	Paper chromatography	.Butoxide fluorescence .Radioactivity	10	Cortisol	17.5 [±] 4.2	100 [±] 14%	Δ^4 -3-ketosteroids
Peterson et al 1957	5	Methylene Chloride	—	.Porter-Silber .Radioactivity	50	17-OH-CS	15 [±] 4.5 6-25	94.5% 96.6%	17,21 dihydroxy- -20-ketone
Peterson 1957	30-40	CCl ₄ / CH ₂ Cl ₂ 4 2 2 1/1	Paper chromatography	.Sulphuric acid fluorescence .Radioactivity	20	Corticosterone	1.1 [±] 3 0.5-2	99%	11-hydroxysteroids
Kalant 1958	1	Dialysis Methanol/CH ₂ Cl ₂	Paper chromatography	Sulphuric or phosphoric acid fluorescence	5 (Dogs)	Cortisol	33 [±] 6	89 [±] 11%	11-hydroxysteroids
Ely et al 1958	5	Ethyl acetate	Florisil then silica gel column chromatography	Sulph. ac. fluorescence	20	Cortisol Corticosterone	10.9 [±] 0.55 3.0 [±] 0.2	64% 61%	11-hydroxysteroids
McLaughlin et al 1958	2-8	Chloroform	Silica gel column chromatography	Sulphuric acid fluorescence	21	Cortisol Corticosterone	12.4 2.0	80-90% 82-84%	11-hydroxysteroids
Silber et al 1958	1	Methylene chloride	—	Sulphuric acid fluorescence					11-hydroxysteroids
Moncloa et al 1959		Methylene chloride	—	Sulphuric acid fluorescence	12 (Rats)	Corticosterone	19.4 13.9-26.8		11-hydroxysteroids
Thomasson 1959	5.5	.Methylene chloride .β glucuronidase hydrolysis	—	Porter-Silber	194	Free & Conjugated 17-OH-CS	19.6 9-32 (Free)	80%	17,21 dihydroxy- -20-ketone
De Moor et al 1960	1-2	Methylene chloride	—	Sulphuric acid fluorescence		11-OH-CS	21.9 [±] 4.67		11-hydroxysteroids

PART 1.

The Simultaneous Fluorometric
Microdetermination
of Cortisol and Corticosterone.

EXTRACTION.

EXTRACTION.

Introduction .

Corticosteroid hormones in the blood although attached to proteins, forming protein steroid complexes, are freely extractable. Extraction from plasma has been carried out in various ways, consisting mainly in:

1. Solvent extraction.
2. Dialysis.
3. Charcoal adsorption.

Extraction by solvents is the most widely used method. The recovery of steroids in a wide range of concentrations is possible, often approaching the theoretical value, and there is little danger of conversion of the steroid into artefacts (45). Chloroform (11,17,21,39,91 123), methylene chloride (29,95), chloroform-ether(66) ethyl acetate (22,41) and isopropyl acetate (111) are superior to the other solvents as the proteins are not precipitated and there is less difficulty from the formation of emulsions (45). The disadvantage with the solvent extractions is the repeated handling of the sample and the significant variation from sample to sample in the amount of emulsion formation (45).

Extraction following dialysis has been used by several investigators (5,62,71,92,145,146). In the method described by Axelrod and Zaffaroni (5), the technique consisted of dialysing the blood sample in a cellophane sausage for 48 hours. The inside phase consisted of one volume each of blood, water and methanol and the outside phase of 30% methanol and chloroform. The advantage of this procedure over the solvent extractions is a minimum of manipulation of each sample which reduces the possibility of losses and of artefact

formation. With polar compounds such as steroids with 4 or 5 oxygen atoms, the yield is reasonably good, the extracts are free from fat, cholesterol and pigments, and a large number of samples can be extracted at the same time under almost identical conditions. However, there are some disadvantages, the recovery is not complete for less polar compounds, the large volume of the organic phase due to the fact that the immiscible solvent must be repeatedly changed, and the method requires 48 hours or more to perform.

Charcoal has been used as an adsorbant for steroids from blood (66,78), primarily in perfusion experiments, the method seems useful when large amounts of blood are extracted, but has the great disadvantage that charcoal frequently produces artefacts and occasionally destroys some of the corticosteroids.

After extraction, most workers wash the plasma extract with 0.1 N sodium hydroxide, potassium hydroxide or sodium carbonate to eliminate non-steroidal formaldehydogenic materials and to remove the phenolic fraction of the extracted steroids (18). The extract is then washed with water, neutralised by adding a few drops of acetic acid, traces of water removed either by adding ethanol towards the end of distillation or by anhydrous sodium sulphate or anhydrous calcium chloride.

METHOD.

The method adopted for extraction employed solvent extraction of the corticosteroids from the plasma by methylene chloride. It included an important modification from the usual methods, that is the addition

of sodium hydroxide to the plasma before extraction (combined extraction and neutral washing). It is described as follows:

Reagents

1. Petroleum ether AR, B.P. 40 - 60 C, obtained from M & B.
2. Methylene chloride AR, ~~B.P.~~, obtained from BDH. It is freshly redistilled.
3. $\frac{N}{20}$ sodium hydroxide solution.

The tubes used for extractions are 10 ml. capacity, ground glass stoppered pyrex (those used for collecting the extract are 50 ml. capacity). They are boiled with steroxol, rinsed thoroughly with hot water then with distilled water and finally with ethanol. This method of washing was proved to be satisfactory by examining the sulphuric acid fluorescence of 10 ml. methylene chloride dried in these tubes. It was found that their fluorescence is within the usual range of the blank fluorescence.

PROCEDURE

1. About 1.2 ml. heparinized plasma is washed once with 5 volumes petroleum ether. The petroleum ether layer is discarded by means of a fine pipette connected to a vacuum suction.
2. To 1 ml. of the washed plasma pipetted into another tube, 1 ml. $\frac{N}{20}$ sodium hydroxide is added. The tube is rotated gently to mix them.
3. The alkaline diluted plasma is then extracted twice with approximately 6 ml. methylene chloride each time by gentle inversion of the well-stoppered tubes 30 times, avoiding vigorous shaking which

- results in emulsion formation. After centrifugation, the extract is transferred into another tube by means of a syringe with a needle long enough to reach the bottom of the extracting tube.
4. The extract is brought to dryness under reduced pressure in a water bath around 30°C. The dry extract is then ready for the next step.

DISCUSSION.

Plasma and not whole blood was used for the extraction because it has been shown that corticosteroids are present largely in the plasma (26,36,84,91). The use of plasma is preferable because it eliminates the question of large amounts of protein from the red cells. Since it has been shown that erythrocytes combine very rapidly and irreversibly with up to 50% of the cortisol and probably with other corticosteroids as well (26,36,101) it is essential that heparinized plasma be separated as soon as withdrawn.

Solvent extraction was preferred on account of being easier and much quicker than extraction by dialysis. Comparing the various solvents used, Peterson et al (95) found that ethyl acetate, chloroform and methylene chloride were the most efficient of the organic solvents for the extraction of corticosteroids. The partition coefficient for cortisol in ethyl acetate, chloroform and methylene chloride was found to be 0.12, 0.13 and 0.14 respectively; while in ethylene dichloride, benzene and ethyl ether, it was found to be 0.28, 0.60 and 1.30 respectively. Methylene chloride has various advantageous characteristics over ethyl acetate and chloroform while all

the three have very much the same efficiency in removing cortisol from water.

Redistilled chloroform is only stable for a few days. Ethyl acetate is difficult to purify and should only be used if its pH is greater than 6. (27). On the other hand, methylene chloride is easy to purify and remains free from impurities for a long time and is stable at room temperature. An additional advantage of methylene chloride over chloroform is that it has a lower boiling point, thus rendering quicker the subsequent step of drying the extract under vacuum.

Methylene chloride was redistilled before use. This was found to lower the blank fluorescence by an average of 20%. It was not purified by passage through column with activated silica gel as advocated by some workers (33) as this was found to have no effect in lowering the reagent blank. Distillation of methylene chloride is strongly recommended because fluorometry is too sensitive a method to allow for any interference by fluorogenic impurities in the reagents.

In 1957, Bondy et al (11) found that the addition of sodium hydroxide to the plasma before extraction was simple, it resulted in a clear extract and had no harmful effect. No destruction of cortisol was attributed to this procedure because the buffering action of the plasma proteins prevented the pH to rise above 9 and because exposure to alkali lasted for only a few minutes.

This claim was tested on 16 sheep's sera, 1 ml. each, divided into two groups after washing with petroleum ether. One group was extracted with methyl-

ene chloride , the extract washed with $\frac{N}{10}$ sodium hydroxide which was then aspirated, the extract was washed with water and dried with 2 gms anhydrous sodium sulphate. The second group was simply extracted with methylene chloride after the addition of alkali. Both extracts were brought to dryness under vacuum then their sulphuric acid fluorescence measured. The blank of the first group was found to be 36% higher than that of the second group. The fluorescence of the serum extracts of the second group were higher by 40%.

Further study to investigate the cause of the high blank fluorescence always encountered in the first group was carried out by studying the fluorescence of the individual constituents of the blank as seen in Table 2.

From this study the following conclusions could be drawn:

1. Methylene chloride raises the fluorescence of the acid blank by 57%.
2. Sodium hydroxide does not contribute any degree of fluorescence to the blank.
3. Sodium sulphate raises the fluorescence of the acid methylene chloride blank by 70% and calcium chloride by 107%.

Therefore the cause of the high fluorescence of the blank of the first group of sera is due to drying the extract with anhydrous sodium sulphate.

It becomes evident therefore that an additional advantage of combined extraction and neutral washing, suggested by Bondy et al, is to avoid the use of the highly fluorogenic sodium sulphate or calcium chloride

as a drying agent. In addition, this simple modification ensures that no corticosteroid is lost in the processes of washing and drying. Such losses, during the repeated handling of the extract could explain the lower fluorescence readings of the first group.

The efficiency of the extraction process was proved by recovery of cortisol averaging 92.67% (range 89 - 98%) . (Table 2) .

11.

PURIFICATION AND FRACTIONATION
OF THE NEUTRAL EXTRACT.

PURIFICATION AND FRACTIONATION
OF THE NEUTRAL EXTRACT.

In the blood, corticosteroids occur in relatively low concentration and in association with other structurally related steroids, a fact which makes their quantitative estimation quite difficult. For this reason, fractionation of the crude neutral extract is necessary as far as possible into qualitatively well defined groups, the specific determination of which can then be carried out.

Chromatographic methods, being efficient and convenient for this purpose, have, therefore, attracted the interest of chemists working in the steroid field. Corticosteroids have been subjected to adsorption chromatography and partition chromatography.

ADSORPTION CHROMATOGRAPHY.

INTRODUCTION.

Adsorption chromatography is suitable for lipid-soluble compounds and has found wide application in the steroid field.

Alumina has been the mainstay of steroid chromatography for nearly 20 years and has been classically used by Reichstein and Shoppee for the separation of adrenal extract (106).

In 1950, Pincus and Romanoff (100) introduced silica gel and in 1952, Nelson and Samuels (91) introduced magnesium silicate-Celite or Florosil columns

for adsorption chromatography of adrenal steroids in order to avoid losses that occur when alumina is used (103).

The principal use of these methods of adsorption chromatography these days is in the rough separation of steroids into groups prior to paper chromatography or in the application of a relatively specific method of determination directly on the separated groups of steroids, which would not be usable with wholly unfractionated extract (91).

Silica gel is particularly useful for the rapid separation of steroids from fat and cholesterol in extracts of blood or plasma (27). Since the Florosil chromatography does not separate the 17-hydroxy from the 17-deoxysteroids (14) while fractional elution from a silica gel adsorption system is capable of affecting such separation (128), it seemed more suitable to our purpose.

Sweat in 1954, (128) introduced a microcolumn method on silica gel for the chromatographic resolution of cortical steroids, which allowed the use of 5 ml. of blood. He purified silica gel with ethanol, chloroform then washed it with water. He insisted that traces of moisture in either the elution mixture or the silica gel should be eliminated because it changed the polarity of the chromatographic column and caused erratic elution of the steroids. The results given by Sweat were difficult to reproduce in other laboratories, probably because silica gel of insufficiently defined activity was used (14). Neher suggested that an accurately measured water addition would remove this difficulty. To obtain

reproducible chromatograms he recommended always to use purified standardised adsorbant and after full activation to add an accurately measured amount of water in order to deactivate to the required degree (87). For the chromatography of corticosteroids on silica gel, better results were obtained by using 20% saturated silica gel with water than with fully activated material (87).

Because of this apparent controversy, it was decided to study the recovery of cortisol through both types of silica gel.

In Sweat's method the elution of steroids was done by ethanol in chloroform, using increasing concentrations of ethanol from 0.5 - 10%. Neher and Wettstein (92) have recommended the elution by chloroform-acetone mixture. This was found to be simpler and to affect removal of substances from the extract which can disturb the subsequent determination of corticosteroids. They found that a chloroform-acetone 1/1 eluate contains the corticosteroid portion.

METHOD.

Apparatus . a very simple column is constructed of a glass tube 0.6 cm. in internal diameter and 25 cm. long with a tapering end.

- tissue paper washed in Soxhlet extractor with methylene chloride, used as a plug.
- a polythene stopper.
- cork ring.
- micropipette with a rubber tube.
- test tubes.

REAGENTS:

- .. Davison Silica Gel 60 - 200 mesh size, grade 95.
- Chloroform AR, obtained from BDH, B.P. 60 - 62°C, redistilled.
- Acetone AR, obtained from BDH, B.P. 56 - 56.5°C, redistilled.

PROCEDURE.

1. Packing : a small piece of tissue paper, the size of a match head, is plugged into the tapering end of the glass tube by a knitting needle. Approximately 2 gms. silica gel are transferred into the column tube by means of a small funnel.
2. Constitution of the column : 3 ml. chloroform-acetone 99/1 are added and the tube is stoppered and inverted several times to constitute the silica gel column and to get rid of the air pockets.
3. Washing : using another 3 ml. of 99/1 chloroform-acetone.
4. Adsorption : the extract is dissolved in 1 ml. chloroform-acetone 99/1, and is transferred quantitatively by means of the micropipette to the silica gel column. This is followed by washing with 3 ml. chloroform-acetone 99/1.
5. Elution : is done with 10 ml. chloroform-acetone, 1/1. The eluate is dried under reduced pressure and temperature around 40°C.

STUDY OF THE FLUOROGENIC CONTRIBUTION
BY THE VARIOUS REAGENTS.

In order to get the lowest blank fluorescence, a study of the fluorogenic contribution by the various reagents used in silica gel chromatography was carried out.

The blank consisted of the methylene chloride extract of water run through a column of silica gel.

Acetone and Chloroform : 10 ml. of the reagent was dried under vacuum at 40° C, 3 ml. 85% sulphuric acid (V+V) was then added, the tube kept in the dark for 60 min, then the fluorescence measured. A comparison between the fluorescence of the crude reagent and after one redistillation is shown in Table 3 .

Conclusions : Distillation of acetone and of chloroform reduces their sulphuric acid fluorescence by about 50%.

Plug : to determine whether the washed tissue paper plug contributes to the fluorescence, columns were constructed replacing the tissue paper plug by a glass wool plug, the fluorescence of chloroform-acetone 1/1 eluate was found to be equivalent to that run through a column plugged with washed tissue paper, indicating that it has no influence on the fluorescence.

STUDY OF THE FLUOROGENIC CONTRIBUTION OF SILICA GEL UNDER DIFFERENT CONDITIONS OF ACTIVATION.

Crude silica gel, fully activated and 20% saturated silica gel with water were studied.

Crude silica gel : taken directly from the tin, without any washing or drying.

Fully activated silica gel : crude silica gel was washed in Soxhlet extractor with acetone, then with chloroform for 8 hours each. It was then washed with distilled water several times then dried in the oven at 120 C then kept tightly closed in a desiccator.

20% saturated silica gel : a known weight of the fully activated silica gel in a beaker was left in a jar exposed to an atmosphere continuously saturated with water vapour. The silica gel was weighed every day until its weight became stationary, indicating that it had become fully saturated with water. The increase in weight represented the amount of water required for full saturation. Accordingly the amount of water required for 20% saturation was worked out, added to fully activated silica gel in a well stoppered tube, mixed thoroughly in a gentle electric shaker. It was then kept in a tightly-stoppered desiccator.

Procedure.

6 columns of each type of silica gel were packed then washed, as described, with redistilled 99/1 chloroform-acetone. 10 ml. redistilled chloroform-acetone 1/1 were run in each column, collected and dried in vacuum at 40° C, then treated with 85% sulphuric acid for development of fluorescence, kept in the dark at room temperature for 60 min, then fluorescence measured.

Results.

In crude silica gel, the fluorescence of the eluting mixture ranged from 11.0 - 14.5 (average 12.3) microphotometric readings; in fully activated silica gel from 8 - 11.5 (average 9.6) and in 20% saturated silica gel from 11 - 16 (average 14.0).

Conclusion : The least fluorescence of the eluting solvent chloroform-acetone 1/1 was obtained when this was run through fully activated silica gel column. When this was compared to the blank (10 ml. chloroform-

acetone 1/1 both redistilled and dried) it was found that practically no fluorescence could be attributed to the fully activated silica gel itself.

STUDY OF THE RECOVERY OF CORTISOL
FROM SILICA GEL AFTER CHROMATOGRAPHY.

From a standard alcoholic solution of cortisol containing 1 ug/ ml, 0.025, 0.05 and 0.10 ml. were adsorbed on columns of different types of silica gel, eluted with 10 ml. chloroform-acetone 1/1. The fluorescence of the dried eluate in 85% sulphuric acid was measured after allowing it to stand for 60 min. in the dark, and was compared with the fluorescence of similar quantities of standard cortisol. Results are shown in Table 4 .

Conclusions : When cortisol was run through 20% saturated silica gel, part of it was lost, and the recovery of small quantities was very irregular. When run through crude silica gel, the recovery was always above 100%. When adsorbed on to fully activated silica gel and eluted with chloroform-acetone 1/1, cortisol showed an average recovery of about 90% with the small as well as with the larger quantities, yet there was sometimes wide individual variation.

Therefore, fully activated silica gel was selected for the study of the recovery of cortisol from water, for the estimation of cortisol in the plasma and its recovery from the plasma.

The Recovery of Cortisol from water.

0.05, 0.10 and 0.20 ug. cortisol were added to

1 ml. water, extracted with methylene chloride then chromatographed on fully activated silica gel and eluted with chloroform-acetone 1/1 as described. The results of the recovery are shown in Table 5 .

Fluorometric assay of 11-oxysteroids in plasma after chromatography on fully activated silica gel.

1 ml. of human plasma was extracted as described before, then chromatographed on fully activated silica gel. The chloroform-acetone 1/1 eluate was dried and its fluorescence in 85% sulphuric acid, measured after 60 min. The blank was the extract of 1 ml. water treated similarly. Standards were ethanolic solutions containing 0.05, 0.10 and 0.20 ug. cortisol dried, then to each of which the blank was added.

This study was made on heparinised plasma from two normal males and a nephrotic male patient. Each plasma was divided into several aliquots to study the reproducibility of the assay technique. Results are shown in Table 6 .

Recovery of Cortisol from plasma

0.02, 0.05 and 0.10 and 0.20 ug. cortisol were added to 1 ml. of human plasma, extracted and run through silica gel as described above. The results are shown on Table 7 .

Conclusion :

The results of estimation of 11-oxysteroids in human plasma were reproducible but the recovery of cortisol from water or plasma run through silica gel was inconsistent and with smaller quantities was very poor.

DISCUSSION.

Silica gel chromatography has the advantage of resolving steroids from substances causing background contamination. It is also effective in fractionating the extract into various groups of steroids (27,111). Sweat (128) has found that it also could resolve microgram quantities of the major steroids from each other, effectively and in conjunction with the fluorometric procedure, could analyse a mixture of 0.25 ug. each of corticosterone and cortisol. However, these results could not be reproduced in other laboratories; Weichelbaum and Margraf could resolve them only twice in 20 attempts with this technique (141).

In our experiments, no attempt was made to separate cortisol from corticosterone in the plasma extract tested. The main aim was, at first, to determine the reproducibility of the technique and the efficiency of the silica gel in the recovery of cortisol. Since the fluorometric assay, in the wave lengths used, is practically specific for cortisol and corticosterone (vide infra), the technique adopted could be considered as an assay for the plasma - 11-oxysteroids.

The results obtained for estimation of 11-oxysteroids in plasma extracted and run through fully activated silica gel were quite reproducible and promising. However, the recovery of cortisol from water and from plasma after chromatography on fully activated silica gel, showed that a considerable amount of the steroid was lost in the process and there was a wide range of variation. Since it has been shown that the method adopted for extraction is quite efficient and has always given reproducible recoveries over 93%, the wide range

of recoveries and the losses encountered after chromatography of the extract on silica gel could not be due to the extraction step.

Sweat (128) has stressed that in using silica gel for analysis of minute amounts of corticosteroids, sufficient attention should be given to many minute details for the procedure to be reliable and reproducible. A possible cause for the erroneous results obtained is the presence of traces of moisture in the elution mixture or in the silica gel. Such traces would change the polarity of the chromatographic column and cause erratic elution of the steroids.

On closer examination of the results of the recovery of cortisol chromatographed on fully activated silica gel, it was observed that the figures of replicates obtained in one day were very similar but varied greatly from those obtained in another day. This variation from day to day could be due to the difficulty in maintaining the silica gel in a fully activated state though it was constantly kept in the desiccator.

It was felt that Sweat's silica gel microcolumn method, though under certain possible, but extremely rigid empirical physical condition, might give reproducible good recoveries of cortisol, yet it does not offer promise as an investigative or diagnostic tool because precise consistent control of adsorption chromatography is virtually impossible.

PAPER CHROMATOGRAPHY.INTRODUCTION.

Partition chromatography has more advantages over adsorption chromatography; notably the use of R_f values for the identification purposes and the completeness of separation for quantitative analysis. The sensitivity and convenience of paper partition chromatography are additional advantages (55). Reproducible results with crude or complex extracts are obtainable with a minimum of time and trouble (76). With paper strip chromatography several samples may be analysed with ease at the same time.

The main problem with the steroids has been to overcome some special difficulties, principally their unfavourable partition coefficient in the common solvent systems and their tendency to be adsorbed by supporting material, even filter paper (24).

Partition method of chromatography for corticosteroids has been concentrated on 3 major lines of efforts:

1. The use of Keiselguhr columns with polar non-polar solvent system in which the stationary polar phase was aqueous ethanol. This has been extensively used by Morris and his colleagues for estimation of adrenal cortical steroids in blood (84), polarography being used for estimation of the contents of the eluate. Though remarkable for the close approximation to ideal behaviour that can be achieved, the method is quite laborious.
2. The use of filter paper impregnated with a non-volatile organic solvent as a stationary phase. This has

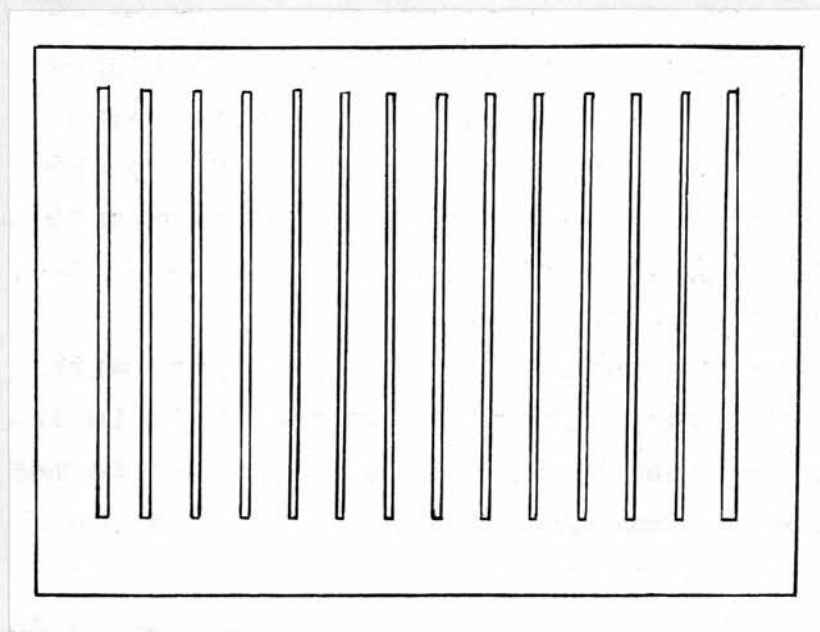
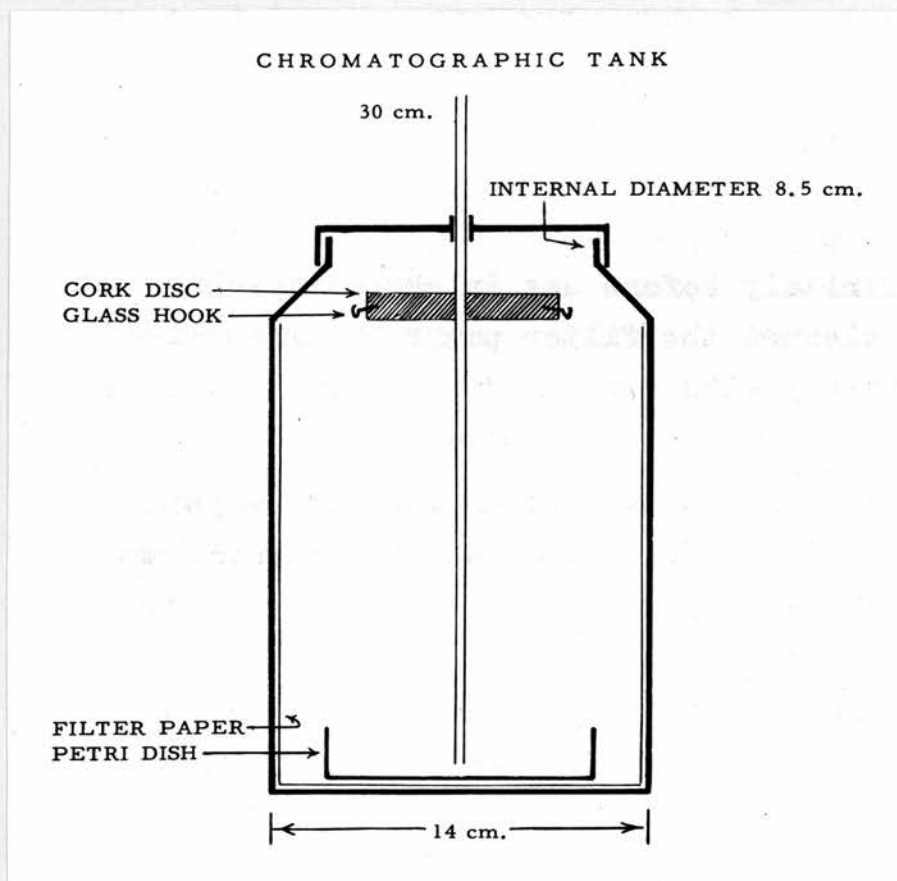
been used successfully for adrenal steroids in extracts from glands, in human urine, in human blood and in adrenal venous blood, by Zaffaroni and coworkers (4,89,115,117,147).

3. The use of orthodox paper chromatography with volatile solvents for both phases. This has been extensively used by Bush and coworkers for analysis of adrenal extract, human urine and blood extracts (21,22,25,27). The separating power of both Zaffaroni and Bush types is very good; Bush type, however, gives more effective separation of cortisol and corticosterone and in a much shorter time.

The choice of the paper chromatographic system must depend on the specific requirement of the problem under study. In the problem for which the present microanalysis is intended Bush's system (benzene-methanol-water system) (21) appeared to be the most convenient. The steroids to be measured were clearly separated in a short run, so that ascending chromatography could be used. This would offer the advantages of more compact zone and more uniform movement of solvent and steroids on the parallel limbs of each chromatogram.

It is well known that established methods of chromatography might fail because extractives from the filter paper might interfere. The interference has been stressed to be particularly serious in the case of spectroscopic procedures for the determination of steroids (55). Various methods had to be devised to remove these interfering substances from filter paper prior to use. As fluorometric methods for determination of corticosteroids are many times more sensitive than spectroscopic procedures, this problem

Figure (1)



Chromatographic Paper

of interfering fluorogenic extractives assumes a great importance.

Lewis (67) has described a capillary washing procedure employing ethanolic sodium hydroxide. He reported that papers cleaned by his method could be stored indefinitely before use in chromatography. Kalant (62) cleaned the filter paper by exhaustive capillary washing with benzene for 24 hours followed by 50% (V/V) methanol for 3 - 4 days.

In the present study a paper chromatographic procedure for the separation of corticosteroids was sought which would be simple enough to permit routine use on a wide scale.

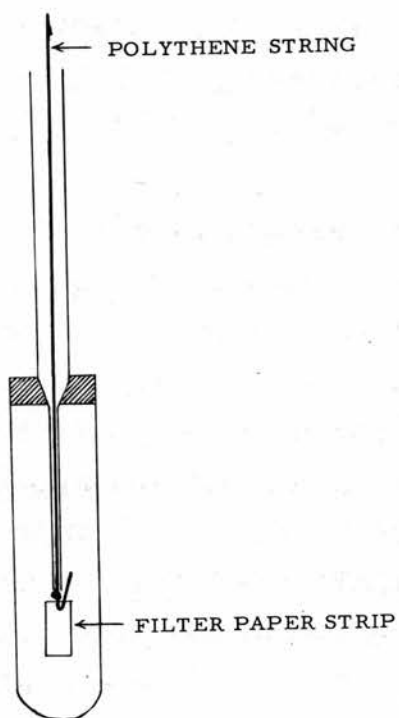
METHOD.

APPARATUS.

1. Paper : Whatman No. 1 paper cut into rectangular pieces 25 x 20 cm. For chromatography each piece is cut into 12 strips 1 cm. wide and 12 cm. long, 3 m.m. apart, and are left joined at the top and bottom to prevent tangling and cross-contamination during handling; the lower joining band is 5 cm. and the upper is 2 cm. (Figure 1).
2. The tank : is a rounded all-glass container with an air-tight cover. Through a central hole in the cover, a glass tube 6 mm. internal diameter is made to reach about 5 mm. from the bottom of the tank.
3. Suspension disc : a cork disc 5 cm. diameter and 1.5 cm. thickness with a central hole is suspended round the central tube about 3 cms. below the cover

Figure (2)

ELUTION OF THE FILTER PAPER



of the tank. Eight short glass rods with tapering upturned ends are fixed equidistant from the periphery of the cork (Figure 1). The interior of the tank is lined by filter paper except for a longitudinal window 6 cm. wide and 30 cm. long. A Petri dish is placed in the bottom of the tank.

Solvents : Benzene, methanol and water 2:1:1 are mixed in a separating funnel. The mixture separates into an upper organic mobile phase and a lower watery stationary phase.

Procedure:

1. The filter paper lining the interior of the tank is wetted by the stationary phase, some of which is also poured in the bottom of the tank avoiding the Petri dish.
2. The extract or the reference steroid is applied to the lower 1 cm. of a separate limb above the broad connecting band by means of a micropipette to one end of which is attached a very fine polythene tube and to the other end a rubber tube to control the flow of the solution during its application. An electric fan helps to dry the points of application.
3. The paper is handled carefully and is hung round the cork disc on the glass suspension hooks in a cylindrical fashion. The tank is closed and equilibration is allowed for 1 hour at room temperature.
4. The mobile phase is poured through the glass tube into the Petri dish until it touches the lower end of the paper. The ascent of the solvent front is observed through the window cut in the lining paper of the tank. At an average room temperature of 23 C, two hours is the usual length of time taken by the solvent front in climbing almost the full length of

TABLE 8

Fluorescence of Whatman no.1 filter paper strips (triplicates)
eluted with absolute ethanol (in 85% sulphuric acid)

Blank microphotometric readings.	1 x 5 cm. Strip		2 x 5 cm. Strip	
	readings	increase	readings	increase
14	30	114%	38.5	175%
14	32.5	132%	39.5	182%
14	32.5	132%	42	200%

TABLE 9

Fluorescence of Whatman no.1 filter paper strips (triplicates)
washed according to Lewis's method, eluted with absolute ethanol
(in 85% sulphuric acid)

Blank microphotometric readings	1 x 5 cm. Strip		2 x 5 cm. Strip	
	readings	increase	readings	increase
12.5	38	204%	46	235%
12.5	44.5	256%	57	314%
12.5	63	404%	69.5	446%

the free limbs of the paper. The paper is then removed and air dried in a ventilated cabinet.

Pure samples of cortisol and corticosterone are run in each chromatogram using one strip for these reference compounds. Another strip is left blank. After chromatography the individual steroid bands are located by ultraviolet light at 240 mμ wavelength. It was found that as little as 1 μg was visible and could be located by this means. This amount, however, will seldom be obtained from 1 ml. of plasma so that the position of smaller quantities were located by analogy with the position of the reference compounds.

Elution : After location of the desired zones by comparison with the pure reference steroids, equal segments bearing the zones are cut from the chromatogram strips for elution. The corresponding segment from the blank strip serves as a reagent blank for the subsequent measurement. Capillary elution was performed by feeding 4 ml. of the eluent on to the paper segment hung by a fine polythene string passing through the capillary tube (Figure 2).

Preparation of the paper for chromatography:

Strips of Whatman No. 1 filter paper 1 x 5 cm. and 2 x 5 cm. were shaken in 8 ml. absolute ethanol in a test tube for ten minutes. The fluorescence of the dried eluate was measured in 85% sulphuric acid after 60 minutes (Table 8).

To reduce the paper fluorescence, various methods of washing were tried and compared.

Washing with ethanolic sodium hydroxide :

The filter paper was washed according to Lewis' method (67), in a large chromatography tank by downward

TABLE 10

Fluorescence of Whatman no.1 filter paper strips (triplicates)
washed according to Kalant's method (in 85% sulphuric acid)

Blank Microphotometric readings	1 x 5 cm. Strip	
	Readings	Increase
17.0	17.0	0
17.0	17.0	0
17.0	17.0	0

TABLE 11

Fluorescence of Whatman no.1 filter paper strips (triplicates)
washed with ethanol or with chloroform-acetone 1/1 (in 85%
sulphuric acid)

Blank Microphoto- metric reading	Absolute ethanol				Blank microphoto- metric reading	Chloroform-Acetone 1/1			
	1st Washing		2nd. Washing			1st. Washing		2nd Washing	
	readings	increase	readings	increase		readings	increase	readings	increas
10.5	20.5	96%	10.5	0	12.0	19.5	75%	12.0	0
10.5	21	100%	10.5	0	12.0	20.5	83%	12.0	0
10.5	22.5	114%	10.5	0	12.0	21.5	92%	12.0	00

syphoning of the following :

1. 2N sodium hydroxide in 95% ethanol for 24 hours.
2. Distilled water until eluate is neutral.
3. 95% ethanol for 3 hours.

It was noticed that after such treatment, the filter paper became crenated and very friable. When eluted with ethanol, similar strips as above, gave a much higher fluorescence than the unwashed paper (Table 9).

Successive washing with the stationary and the mobile phase:

The paper was cleaned as described by Kalant (62) by exhaustive capillary washing with benzene for 24 hours, followed by 50% (V/V) methanol in water for 3-4 days (Table 10).

Simpler methods of washing :

Strips of 1 x 5 cm. paper were washed twice by shaking them vigorously for 10 minutes in a test tube with 4 ml. ethanol. Other strips were similarly washed with Chloroform-acetone 1/1 (Table 11).

Conclusion : The above results confirmed that filter paper contains fluorogenic material that could be eluted with ethanol. Washing the paper with both the mobile and the stationary phases as well as with absolute ethanol or chloroform-acetone 1/1 mixture, at least twice, were equally efficient in cleaning the paper from this interfering fluorescent material.

Big sheets of Whatman No. 1 filter paper were therefore washed with ethanol in a soxhlet extractor continuously for 48 hours, then dried in ventilating cabinet. These washed sheets, together with sheets washed according to Kalant's method were used for further studies.

TABLE 12

Recovery of cortisol run in ethanol washed paper and eluted with absolute ethanol

Amount of Cortisol added in micrograms	Percent Recovery (triplicates)
0.10	21 30 40
0.25	14 26 27
0.50	34 50 54

TABLE 13

Recovery of cortisol from ethanol washed paper eluted with 80% ethanol
and 80% methanol without run.

Amount of Cortisol added in micrograms	80% Ethanol 2-Redistilled Percent recovery	80% Methanol 2-Redistilled Percent Recovery
0.05	110 116 118	85 114 124
0.10	75 85 92	83 88 92
0.15	91 92 92	83 80 93

Recovery of Cortisol run in paper :

0.10, 0.25 and 0.50 microgram cortisol were applied to washed paper (with ethanol), equilibrated for one hour and run for about two hours. Cortisol zones were eluted with 8 ml. absolute ethanol. Results are shown in Table 12.

These low recoveries might be due either to loss of some cortisol during its run in the paper or to its incomplete elution with the solvent used, or to both factors.

Elution of cortisol applied to paper without run, with 80% ethanol and 80% methanol was then carried out. Results are shown in Table 13 . These results have shown that 80% methanol and 80% ethanol were equally efficient in eluting the applied cortisol from the paper. The recovery averaged 95.5% (range 75 - 124%).

0.02, 0.03, 0.05, 0.075 and 0.10 microgram cortisol were then applied to two sets of paper, one washed with ethanol and the other with benzene and 50% methanol as described above. After the run and the elution with 80% ethanol the recoveries were calculated (Table 14).

Conclusion :

When cortisol was applied to the washed paper and eluted without run, with 80% ethanol, its recovery was almost complete. When it was run in ethanol-washed paper, its apparent recovery was always above 100% indicating that fluorogenic material from the paper was eluted together with the steroid. The smaller the amount of the steroid the greater was the faulty recovery. When cortisol was run in benzene-methanol washed paper, its recovery was incomplete especially with smaller amounts.

TABLE 14

Recovery of cortisol run in washed papers and eluted with 80% ethanol.

Amount of Cortisol added in micrograms	Ethanol washed paper	Benzene-methanol washed paper
	Percent recovery	Percent recovery
0.02	205	30
	220	35
	300	35
0.03	126	50
	147	43
	153	40
0.05	240	58
	114	62
	108	70
	106	
0.075	117	53
	120	64
	123	60
0.10	111	69
	117	75
	123	63

DISCUSSION.

Since the final technique of measurement (fluorometry) is not strictly specific for corticosteroids, the cleaning of the paper for chromatography as well as purification of reagents and solvents, are of the utmost importance in order to avoid contaminants which exceed the steroid in amount.

Washing the paper with ethanolic sodium hydroxide as recommended by Lewis (67) resulted in friability and crenation of the paper and in a marked rise in the fluorescence of its ethanolic eluate. It seems that 2 N sodium hydroxide was too strong for washing the filter paper and might have produced chemical alteration responsible for the results observed.

Washing sheets of paper with the mobile and the stationary phases was very efficient in cleaning them from practically all fluorogenic contaminants. Paper strips washed with absolute ethanol were also efficiently cleaned; however, when big sheets of paper were washed, then cut into strips for chromatography, high recovery results were obtained. This showed that fluorogenic contaminants from the paper could still be eluted indicating that big sheets of paper were not as thoroughly cleaned as the small strips were.

Recovery of the microgram amounts of cortisol run in the benzene-methanol washed paper resulted in loss of part of the steroid. The loss was greater with smaller amounts. A possible explanation for the poor recovery of such small amounts is that the zones were too faint to appear in ultraviolet light, and were located by comparison with the position of reference steroid. Such location might not be accurate enough to include all the steroid. Some loss of the

steroid during its run might account in part for the low recovery results.

For paper chromatography to give fully reproducible results for steroids to be assayed fluorometrically, a series of precautions must be observed scrupulously. The requirements are so stringent that paper chromatography seemed unlikely to be suitable for routine use on a large scale. Moreover, very small amounts of cortisol were partly lost during the run in paper and so it did not appear promising for a microanalysis technique. Therefore, it was decided to proceed to study the phenomenon of sulphuric acid fluorescence of cortisol and corticosterone to find out the optimum condition which selectively favours these two steroids when both are present in a mixture, thus avoiding the necessity of their separation by paper chromatography prior to their quantitative estimation.

111.

FLUOROMETRY.

FLUOROMETRY.Introduction.

It has been known for many years that a number of steroids when treated with concentrated sulphuric acid, phosphoric acid or other acids, exhibit visible fluorescence under suitable illumination. This phenomenon has been adapted quantitatively for fluorophotometric measurement of various estrogenic steroids (6) but much less attention has been directed to the quantitative possibilities of the fluorescence shown by adrenocortical steroids in acid solution.

Interest in the phenomenon of fluorescence of corticosteroids was revived by the finding of Neher and Wettstein (88) that all physiologically active corticosteroids when isolated on paper chromatograms, fluoresce with distinctive colours under ultraviolet illumination after the chromatogram has been heated with sulphuric acid, phosphoric acid or acetic anhydride. The fluorescence phenomena have been utilised in the development of qualitative tests for the detection of various steroids and in fluorophotometric procedures for their measurement in extracts of biological materials. Finkelstein in 1952 (40) developed a quantitative fluorophotometric assay for certain corticosteroids dissolved in 85% phosphoric acid with the aid of heat. Sweat, in 1954, (127) reported a more sensitive method in which the steroids were dissolved in concentrated sulphuric acid at room temperature. Both authors found the method to be applicable only to corticosterone and cortisol, and, at a considerably lower

level of sensitivity, to 11-deoxycorticosterone. In 1958, in a thorough study of the fluorescence phenomenon of corticosteroids, Kalant (60) confirmed that a wide range of steroids could fluoresce in concentrated acids, and that the specificity of sulphuric acid fluorescence described by Sweat (127), Goldzieher et al (48) and others was valid only with respect to the reaction conditions described by these authors.

Very little is known about the nature of the steroid-acid reaction and about the number and identity of the products resulting from it. Zaffaroni (145) demonstrated the rapid formation of a $\Delta^9 - 11$ dehydration product when corticosterone was dissolved in concentrated sulphuric acid. However, Neher and Wettstein (88) showed that fluorogenic products could be obtained by the action of phosphoric or sulphuric acids on other steroids which could not possibly undergo the same internal dehydration.

In 1958, Kalant (60) pointed out that the mechanism of reaction of a given steroid is essentially the same in phosphoric acid or sulphuric acid, differing in rate rather than in nature. He tried to explain it on the basis of a three-stage-reaction pattern; reversible protonation of Δ^4 -C-3 ketone of corticosteroids, followed by intermolecular association complexes at substituent reactive sites of other steroid molecules. These complexes were thought to break down irreversibly to yield a series of products via reactions which were markedly affected by dilution.

Whatever the nature of the reaction may be, it seems



that the fluorescence intensity is strongly affected by the number of hydroxyl groups in the steroid molecule (48,127). The steric configuration of the group is important; alpha hydroxyl is usually more fluorogenic than the beta isomer (48). The α/β -unsaturated ketone in ring A also exerts some influence on the fluorescence; saturation of this group in the 11-hydroxycorticosteroids results in marked reduction in the intensity of the fluorescence produced (127).

The intensity of the fluorescence emission is a function of the amount of energy trapped by the fluorescent molecule. It depends, therefore, not only on the intensity of the source of the exciting radiation, but also on the efficiency with which this radiation is absorbed. Thus both the characteristic of the light source and the absorption spectrum of the irradiated solution are important factors in achieving maximum excitation efficiency (49).

Both the absorption and the fluorescence phenomena are influenced by acid concentration, time and temperature of incubation and other reaction measurement conditions (49,61). The various factors that affect the absorption spectra of steroids dissolved in concentrated acids have been reasonably fully investigated. Zaffaroni (144) had shown that the absorption spectrum of any steroid dissolved in concentrated sulphuric acid underwent progressive change with the passage of time. Umberger and Curtis (137) had demonstrated changes dependent on the concentration of the acid used. Linford and co-workers (128,129) carried out detailed studies on the effect of time and temperature and on dilution of the reaction mixtures with alcohol or with

water on the absorption spectra of the steroid acid reaction mixture. Much less attention has been paid to the effect of changing the reaction condition on the degree and the development of the fluorescence.

Because of the potential advantages inherent in the great sensitivity of fluorometric methods of measurement of steroids, it seemed worth-while to explore further the various conditions which affect the development of the fluorescence when steroids are treated with sulphuric acid. It was hoped that if such conditions were known, greater sensitivity and specificity of fluorometric measurement might be achieved by purposeful direction of the reaction. The results presented here have led to a partial fulfilment of the hope for a simple method for quantitative fluorometry applicable to 1 ml. of plasma.

METHOD.

Apparatus :

Aminco-Bowman spectrophotofluorometer, with a high intensity xenon continuum light source. This makes available ultraviolet activation wavelengths other than those provided by the usual sources. The photomultiplier tube provides a sensitive detector of the emitted fluorescent light. Increased versatility is provided by a system of two monochromators (one for activation light, one for fluorescent light) which display either the activation spectrum or the fluorescent spectrum. This feature permits one to obtain equivalent absorption

Figure (3)

FLUORESCENCE SPECTRUM OF CORTISOL AT DIFFERENT ACTIVATION WAVELENGTHS

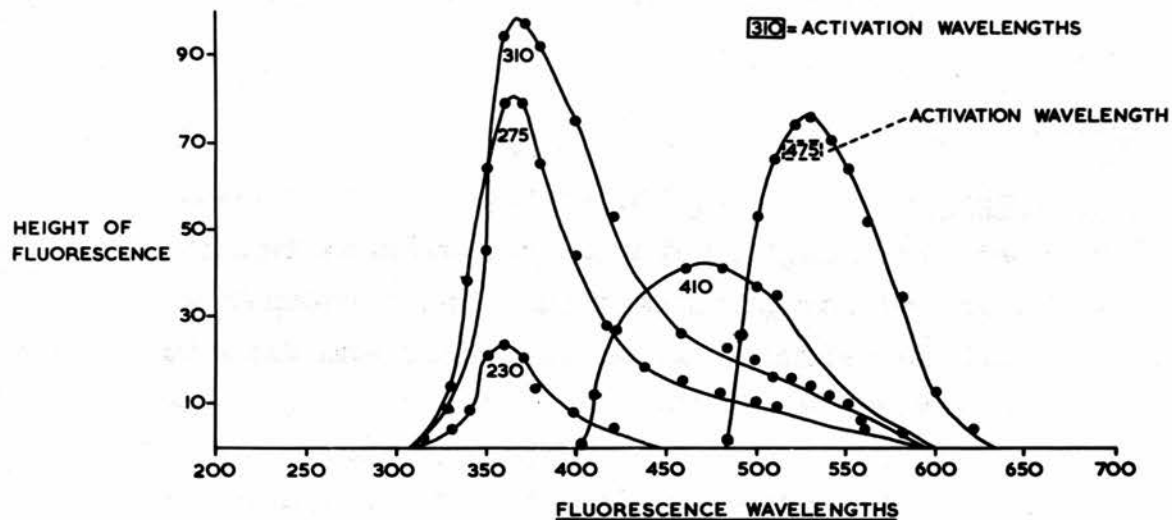
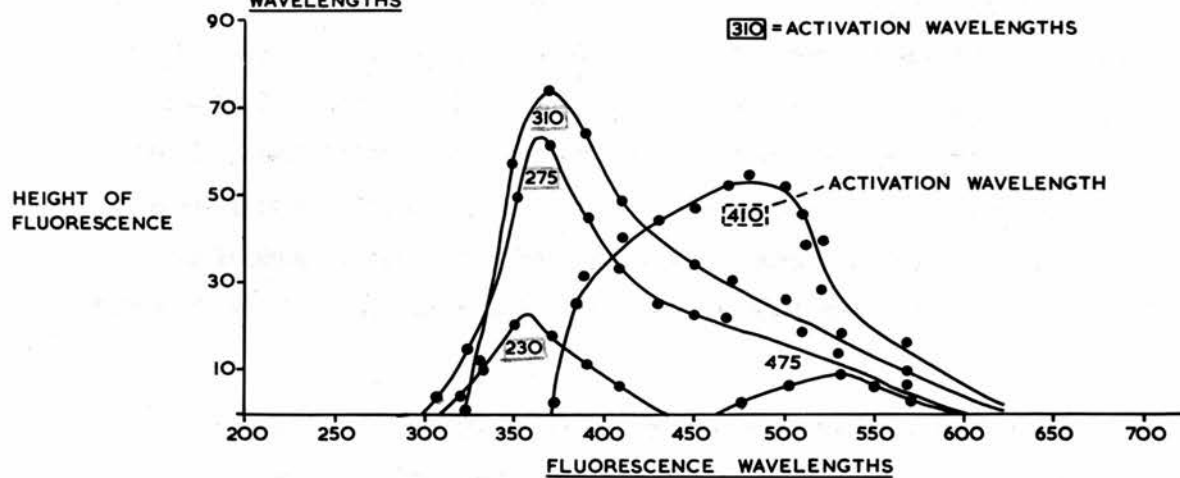


Figure (4)

FLUORESCENCE SPECTRUM OF BLANK AT DIFFERENT ACTIVATION WAVELENGTHS



characteristics at concentrations which are lower in magnitude than those required by spectrophotometry.

4.8 ml. fused quartz cells were used. They were washed thoroughly with distilled water and dried with ethanol.

Reagents :

1. Sulphuric Acid : AR. 98.08%, S.G. 1.84, it was obtained from B.D.H. and was used without redistillation or further purification. For convenience, this will be referred to as 100% acid and dilution will be designated by reference to it, thus three parts by volume of the concentrated acid mixed with one part of distilled water will be considered as 75% (V + V) sulphuric acid.
2. Ethanol : was twice redistilled before use.

Determination of the Activation Wavelength and the fluorescence Wavelength for Corticosterone and Cortisol.

Standard ethanolic solutions containing 0.04 microgram corticosterone per ml. and 0.20 microgram cortisol per ml. were used. 1 ml. of each standard was pipetted in a test tube, dried under reduced pressure, then 0.20 ml. ethanol added. 1.8 ml. 75% (V+V) sulphuric acid was then added, mixed thoroughly and left in the dark for 60 minutes. The samples were transferred into the quartz cells.

The apparatus was set at sensitivity 50, meter multiplier 0.03 and the filter was removed. The fluorescent wavelength disc was turned to the shortest wavelength

TABLE 15

Comparative fluorescence of 0.10 microgram of various steroids
in 75% (V+V) Sulphuric acid after two hours at room temperature.

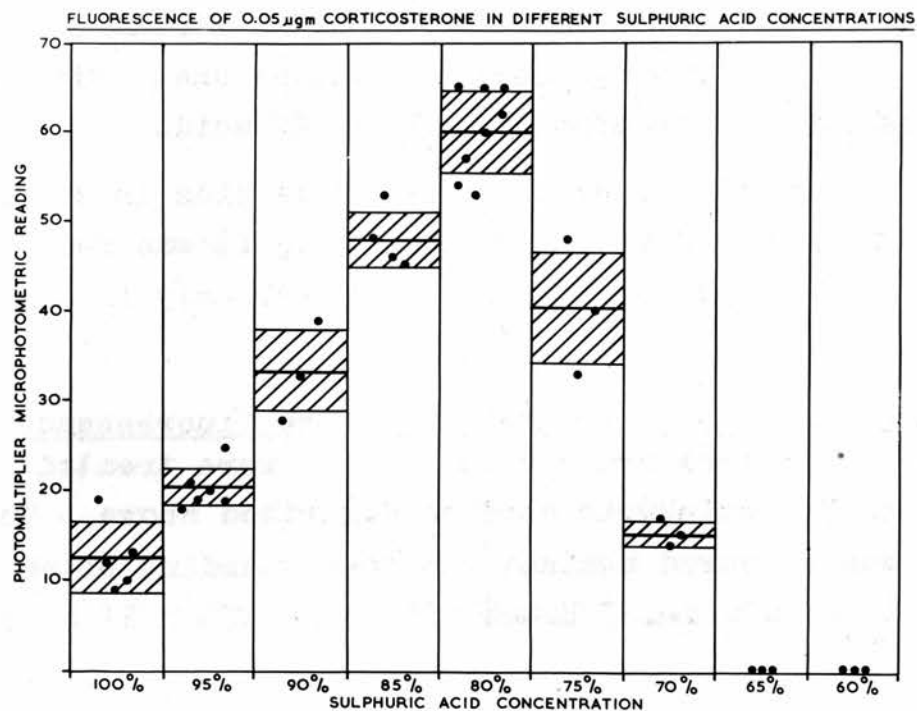
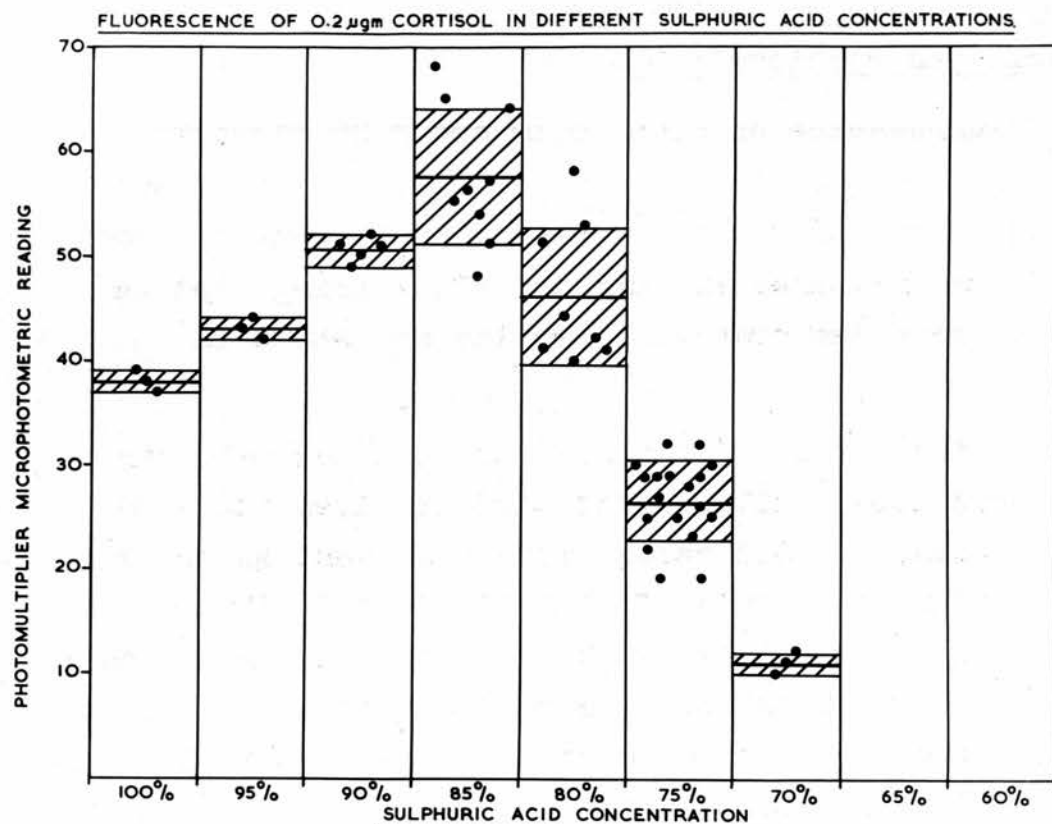
Steroid	Units of fluorescence
Corticosterone	100
Cortisol	23
Cortisone	2.5
Tetrahydrocortisol	0
Tetrahydrocortisone	0
17-hydroxycortexone	7
17-hydroxyprogesterone	6.5
Deoxycortone acetate	5.5
9- α -fluorocortisol	3
Prednisone	0
Dexamethasone	0
Methandrostenolone	2
Cholesterol	1

(200 mu), and was moved manually in steps of 20 mu at the completion of each activation scan. The activation spectrum for each fluorescent wavelength was recorded on the drum. The height of the recorded fluorescence at each activation wavelength was measured and plotted against the corresponding fluorescent wavelength, (Figure 3). The blank was treated similarly (Figure 4). Five activation wavelengths were found capable of exciting a fluorescence; - 230, 275, 310, 410, and 475 mu. The emitted fluorescence resulting from excitation by the first four of the activation wavelengths was almost identical in height and in location in both steroids tested and in the blank. At 475 mu activation, the maximum fluorescence was obtained at 530 mu for both steroids. The fluorescence of the blank at these particular wavelengths was found to be 15% that of cortisol. Therefore, at an excitation wavelength of 475 mu the maximum emitted fluorescence at 530 mu is due to the steroids. These wavelengths were thus selected in all the subsequent studies.

Specificity :

To investigate the specificity of the fluorescence at the selected wavelengths to cortisol and corticosterone, several steroids in quantities of 0.10 microgram per ml. were treated with 75% sulphuric acid and their fluorescence measured with 475 mu exciting wavelength and 530 mu emitted wavelength, 120 minutes after mixing, at room temperature. The blank was set at zero reading and 0.10 microgram corticosterone at 100 for comparison. The machine was set at meter multiplier 15 , sensitivity 0.003 and OG 1 green filter was used. Results are shown in Table 15. It can be seen that cortisol gives a fluorescence equal to 23% that of corticosterone while the other steroids give negligible fluorescence in the above specified condition.

Figure (5)



The effect of acid concentration on the fluorescence of cortisol and corticosterone.

The fluorescence of 0.05, 0.10 and 0.20 microgram cortisol and 0.02, 0.04 and 0.05 microgram corticosterone in sulphuric acid of varying concentrations ranging from 60 - 100%, was measured 120 minutes after being kept in the dark at room temperature. Results are shown in Figures 5, 6 & 7.

It was found that cortisol fluoresces maximally in 85% sulphuric acid, while corticosterone gives its maximum fluorescence in 80% acid. This was found to be true with the different amounts of steroids tested, thus indicating that it is a characteristic phenomenon of the steroid itself under the above specified condition. In general, cortisol fluorescence was high in high acid concentrations and although it was maximal in 85% concentration, yet its fluorescence in 100, 95, 90 and 80% was nearly as high. With corticosterone, on the other hand, there was a good difference between its maximum fluorescence in 80% and that in other acid concentrations. Neither steroid showed any fluorescence in 65 or 60% acid.

When the activation spectra of both steroids in these different acid concentrations were recorded, it was found that they all had the same shape and differed only in magnitude.

The Effect of Time on the development of the fluorescence :

Samples of cortisol and corticosterone were treated with 85, 80 and 75% sulphuric acid as described above. The fluorescence was measured against the corresponding blank at 5 minutes intervals for 3 hours and again after 24 hours.

With 85% sulphuric acid cortisol fluorescence was found

Figure (6)

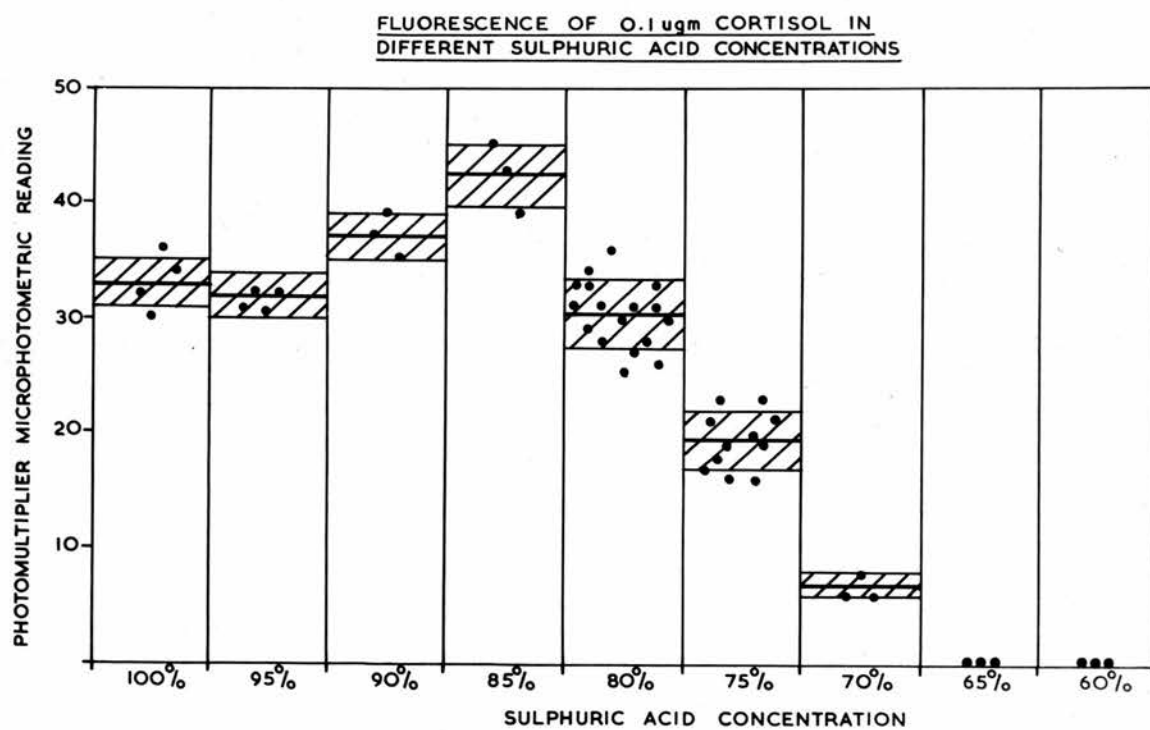
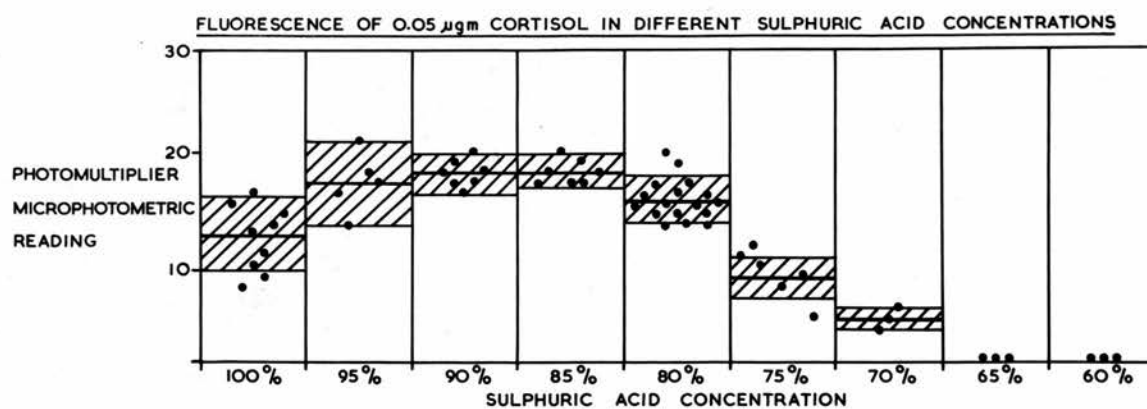
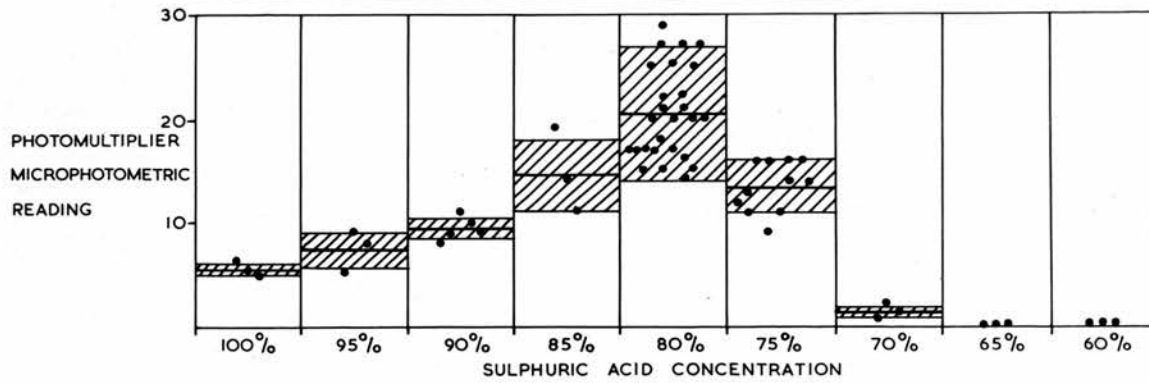


Figure (7)

FLUORESCENCE OF 0.02 μ g_m CORTICOSTERONE IN DIFFERENT SULPHURIC ACID CONCENTRATIONS



FLUORESCENCE OF 0.04 μ g_m CORTICOSTERONE IN DIFFERENT SULPHURIC ACID CONCENTRATIONS

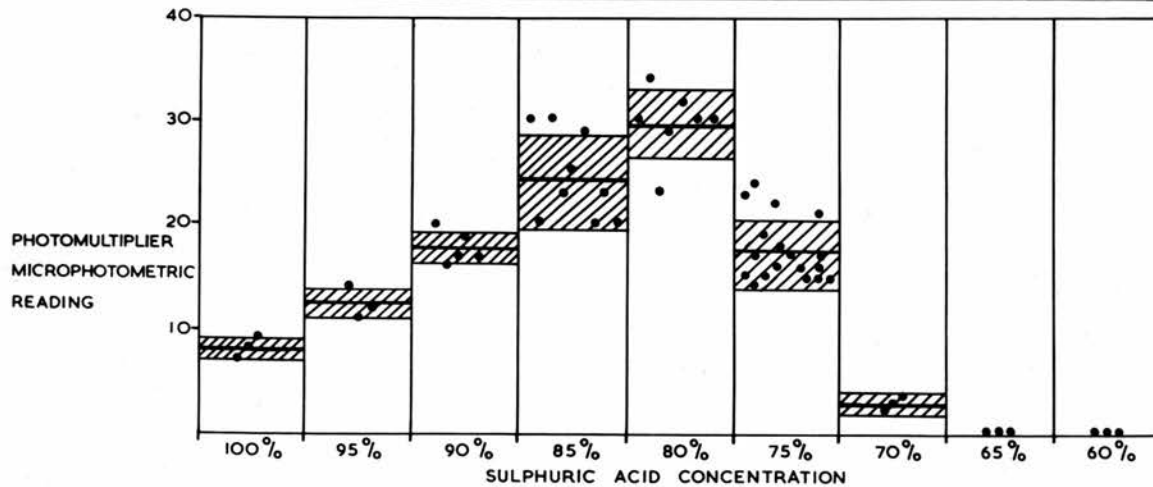


TABLE 16

Comparison between the fluorescence of cortisol and corticosterone at 23°C and 37°C.

ug. Cortisol	Units Fluorescence		µg. Corticosterone	Units fluorescence	
	23°C	37°C		23°C	37°C
0.05	16	17	0.02	24	13
0.10	40	38	0.05	60	33
0.15	57	57	0.10	109	62
0.20	71	79			

to reach its maximum 10 minutes after mixing, while corticosterone took 30 minutes. With 80% acid, cortisol reached its maximum after 30 minutes and corticosterone after 60 minutes. With 75% acid cortisol reached the maximum after 45 minutes and corticosterone after 75 - 90 minutes. The fluorescence after its full development remained steady for two hours. No appreciable change was noted after 24 hours.

The effect of temperature on the fluorescence:

- A. Boiling : the fluorescence of 0.05, 0.10 and 0.15 microgram in 85% acid was measured against the blank after boiling for 10, 20, 30 and 60 minutes. It was observed that the blank fluorescence was greatly increased by boiling. Readings of the standard's fluorescence under this condition of boiling was impossible because of such high blank.
- B. Heating to 50°C : The fluorescence of 0.20 microgram cortisol and 0.04 microgram corticosterone in 80, 70 and 60% sulphuric acid was studied after incubation at 50°C and measured every 30 minutes for 3 hours and compared with the corresponding fluorescence at room temperature. It was found that the blank fluorescence, in the three acid concentrations used, got progressively higher the more the duration of heating. In 70 and 80% acid cortisol fluorescence was higher than that developed at room temperature, while corticosterone fluorescence was diminished. In 60% acid neither steroid fluoresced even after heating for 3 hours.
- C. Incubation at 37°C : The fluorescence of 0.05, 0.10 and 0.15 microgram cortisol, 0.02, 0.05 and 0.10 microgram corticosterone in 85% acid incubated at 37°C for 60 minutes was compared with the corresponding fluorescence at room temperature. The results are shown in Table 16. The blank did not show any rise after incubation at 37°C for 60 minutes. These results

TABLE 17

The fluorescence of a known mixture of cortisol and corticosterone in sulphuric acid after 60 minutes at 23°C compared with that of the individual steroids.

Acid conc.	Corticosterone (A)		Cortisol (B)	Mixture	Sum of (A) + (B)
	0.02	0.05	0.10		
100%	7		36	45	43
100%		15	36	51	51
95%		20	32	53	52
90%		35	39	77	74
85%	15		42	57	57
85%		48	45	95	93
85%		50	44	93	94
80%		50	34	95	94
80%		58	35	92	93

indicated that heating cortisol at 37° did not change its fluorescence from that developed at room temperature. While when corticosterone was similarly treated, its fluorescence was significantly decreased.

Conclusion : From these studies it could be concluded that under two reaction conditions, the fluorescence of cortisol and corticosterone behaved distinctly differently.

Different acid concentrations or different temperature of incubation were required for optimum demonstration of individual fluorescences. It is possible, therefore, that selective suppression of one or other fluorescent species may be achieved. If this proves true, it may be possible to select suitable conditions for fluorometry of each corticosteroid in a mixture without the need of previous separation.

Estimation of cortisol and corticosterone in a mixture using two different concentrations of sulphuric acid.

To investigate the possibility of utilising differences in the fluorescence characteristics of cortisol and corticosterone in different concentrations of sulphuric acid as a basis for their differential analysis the fluorescence of a mixture of both steroids under different reaction conditions was compared with the fluorescence of the individual steroids under the same conditions. Results are shown in Table 17. The fluorescence of the two steroids was found to be additive in the different acid concentrations used.

The simultaneous determination of the two steroids in a mixture was accomplished by choosing two concentrations of sulphuric acid each giving different relative weights to the fluorescence of cortisol and corticosterone. The quantities of cortisol and corticosterone present could

TABLE 18

Corticosterone		Cortisol	
Micrograms added	percent recovery	micrograms added	percent recovery
0.02	92	0.05	110.6
0.05	107	0.05	95
0.05	106	0.05	89.4
0.05	95.4	0.05	105
	Average 100%		Average 100%

be calculated by substitution in the following set of simultaneous equations :

$$x = \frac{f'A - fC}{f'b - fb'}$$

$$y = \frac{bC - b'A}{f'b - fb'}$$

Where

A,C Fluorescence of the steroid mixture in 80 and 85% acid respectively.

f,f' Fluorescence of 1 ug cortisol in 80 and 85% acid respectively.

b,b' Fluorescence of 1 microgram corticosterone in 80 and 85% acid respectively.

x micrograms corticosterone in the mixture.

y micrograms cortisol in the mixture.

This theoretically sound principle was then practically tested. A mixture of 0.02 microgram corticosterone and 0.05 microgram cortisol was treated with 100 and 85% sulphuric acid, and a mixture of 0.05 microgram corticosterone and 0.05 microgram cortisol was successively treated with 100 and 80%, 95 and 80% and 90 and 80%, sulphuric acid. The fluorescence of the samples was measured 60 minutes after mixing. Standards of corticosterone and cortisol were treated similarly in each group. Results are shown in Table 18.

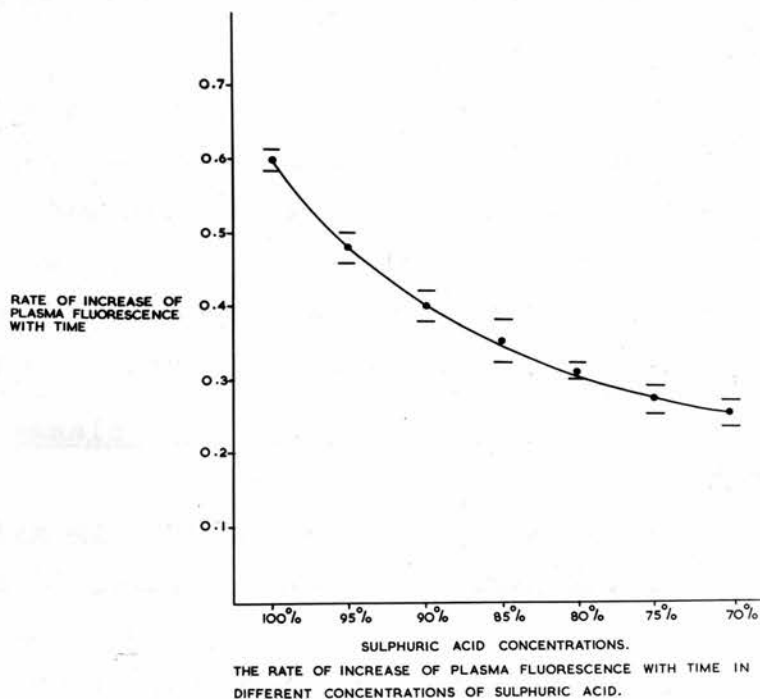
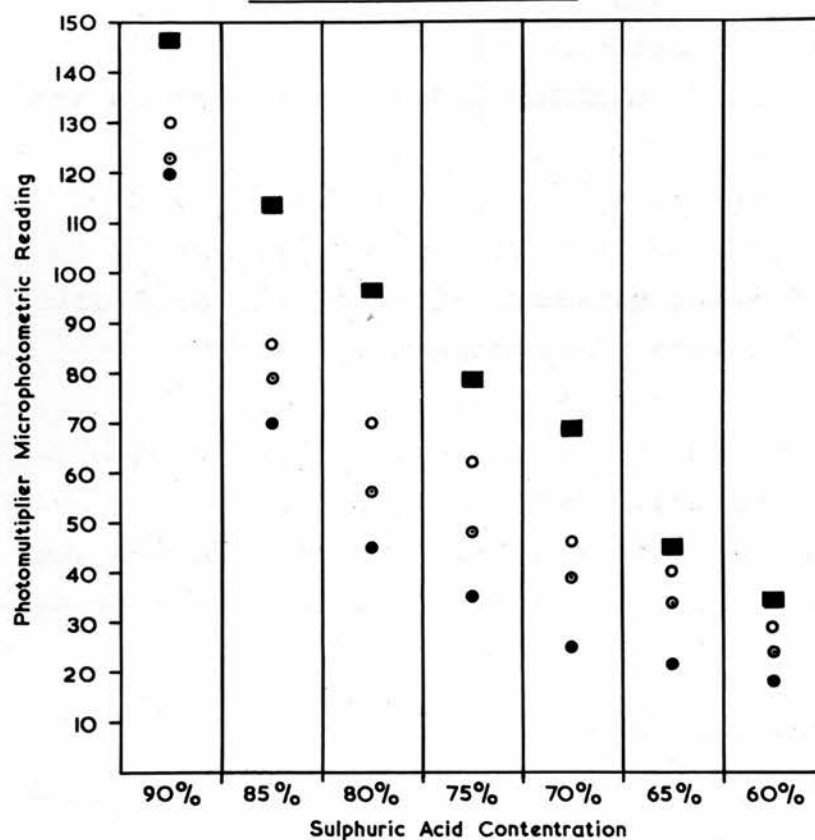
Study of the fluorescence of plasma extract.

(A) In different concentrations of sulphuric acid :

4.5 ml. sheep's serum were extracted and the extract

Figure (8)

Fluorescence of four sheep's plasma extracts in different sulphuric acid concentration



divided into 9 aliquots. The fluorescence in 9 different concentrations of sulphuric acid ranging from 60 - 100% (V+V) was measured 30 minutes after being kept in the dark at room temperature. The experiment was repeated on 4 different sheep's sera. Figure 8 shows the height of the fluorescence of sera in mm. as recorded on the drum in the different concentrations of the acid used. In all the four sera, the fluorescence in 100% and 95% was so high that it could not be recorded on the drum when the instrument was set at sensitivity 40 and meter multiplier 0.01 which are the usual settings used in all the previous studies. The sera, however, did fluoresce in 60% and 65% acid and the fluorescence got progressively higher as the acid concentration increased.

(B) After incubation at 50°C :

The fluorescence of sheep's serum extract in 80%, 70% and 60% was measured after incubation at 50°C for 20 minutes, then every 30 minutes for 3 hours, and compared to the corresponding fluorescence left to develop at room temperature.

In 80% and 70% the reading of the incubated samples were out of the recording scale after 20 minutes incubation. In 60% acid the reading of the incubated sample was double that of the sample kept at room temperature after 20 minutes incubation, and its rate of fluorescence was more rapid than the corresponding sample left at room temperature.

(C) The effect of time on the development of plasma fluorescence :

The fluorescence of sheep's serum extract in different concentrations of sulphuric acid was measured 30 minutes after adding the acid, then every 15 minutes for 3 hours. It was observed that, in all the acid concentrations, the fluorescence of the extract continued to show a progressive rise throughout the 3 hours period. The rate of this

increase with time was greater the higher the concentration of the acid used as seen in Figure 8, which summarises the average and the standard deviation of 28 different experiments on sheep's sera.

Conclusion :

The above studies have revealed prominent differences between the behaviour of the plasma on the one hand and that of cortisol and corticosterone on the other hand in the development of fluorescence under different reaction conditions of acid concentration, temperature and time.

1. The plasma fluorescence was highest in concentrated sulphuric acid, while cortisol fluorescence was best in 85%, and corticosterone in 80%.
2. While cortisol and corticosterone did not fluoresce at all in 60% sulphuric acid, even after heating, the plasma did fluoresce in this acid concentration.
3. The plasma fluorescence continued to rise with time for 3 hours, while both cortisol and corticosterone fluorescence once fully developed remained steady.

From these observations, it could be deduced that the plasma extract contains a fluorogenic substance other than cortisol and corticosterone which would interfere with any attempt to estimate them directly and had to be eliminated.

Elimination of the interfering fluorescence in the plasma utilising the rate of development of the fluorescence :

The development of the plasma fluorescence, with time, in 75% sulphuric acid was more thoroughly studied and compared with the behaviour of cortisol and corticosterone standards in the same acid concentration.

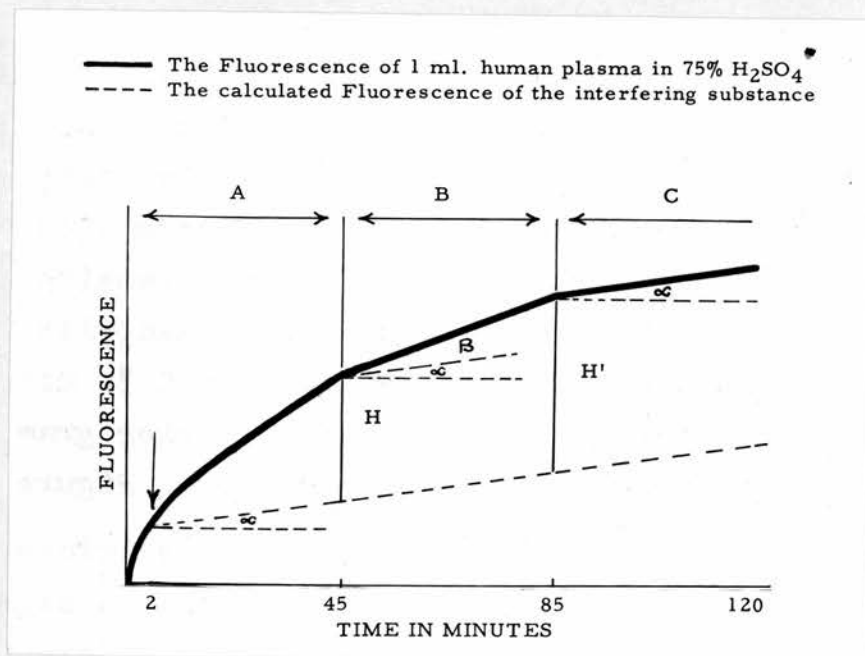
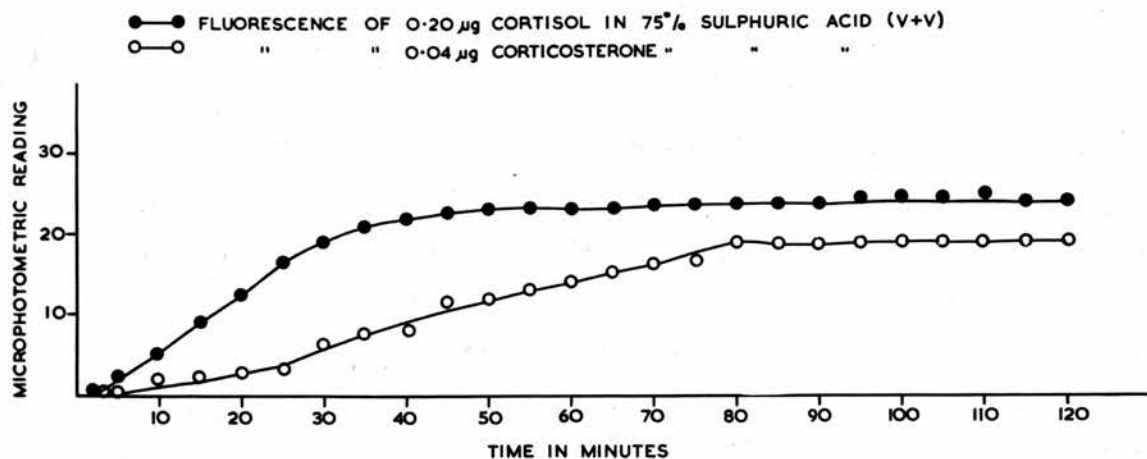


Figure (9)

The fluorescence of the plasma and both standards was measured 2 minutes exactly after mixing with the acid, at 5 minutes, then every 5 minutes for 120 minutes keeping the cuvettes in the dark at room temperature in between the readings. A curve was plotted representing the development of the fluorescence of the plasma extract and both standards (minus the blank) during 120 minutes, Figure 9 . It was noticed that, in this particular acid concentration, at room temperature (20 - 23 C) and at 2 minutes, while both cortisol and corticosterone standards did not show any fluorescence yet, the plasma extract did fluoresce immediately after adding the acid.

It was also noticed that the curve of the plasma fluorescence could be divided into 3 segments :

- (A) A steep rise from the moment of adding the acid till 45 minutes.
- (B) A moderate rise from 45 - 80 minutes.
- (C) A slight rise from 80 - 120 minutes.

Cortisol fluorescence began to develop after 5 minutes and reached its maximum in 45 minutes, while corticosterone fluorescence was rather late and slow in development; it reached its maximum level after 80 minutes. The fluorescence of the two steroids once fully developed remained steady (Figure 9). This phenomenon was tested with different amounts, 0.05, 0.10, 0.15, 0.20 and 0.30 microgram cortisol, and 0.02, 0.04, 0.08 and 0.10 microgram corticosterone, and it was found to hold good (Figure 10).

On the basis of these observations, it was evident that the rise in the fluorescence of the plasma in segment (C) could not be due to either cortisol or corticosterone as the fluorescence of their standards was steady during

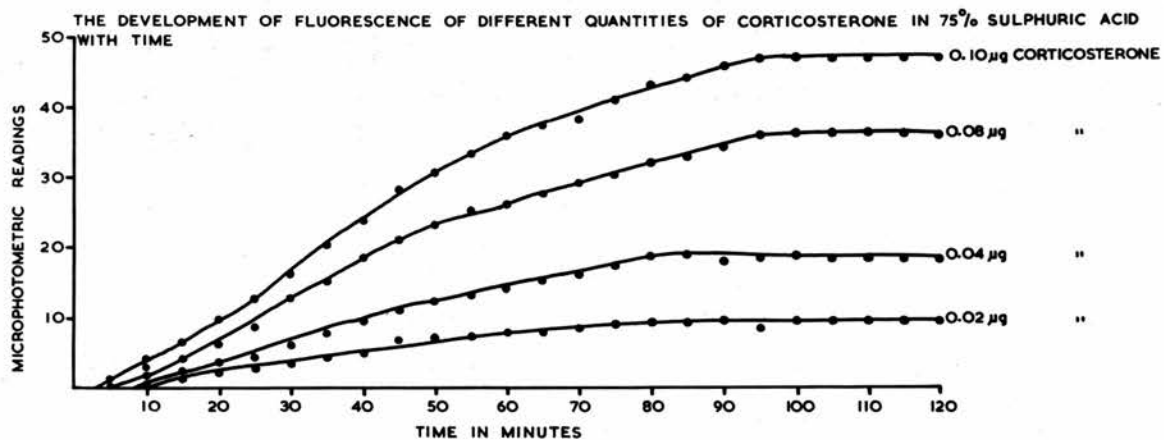
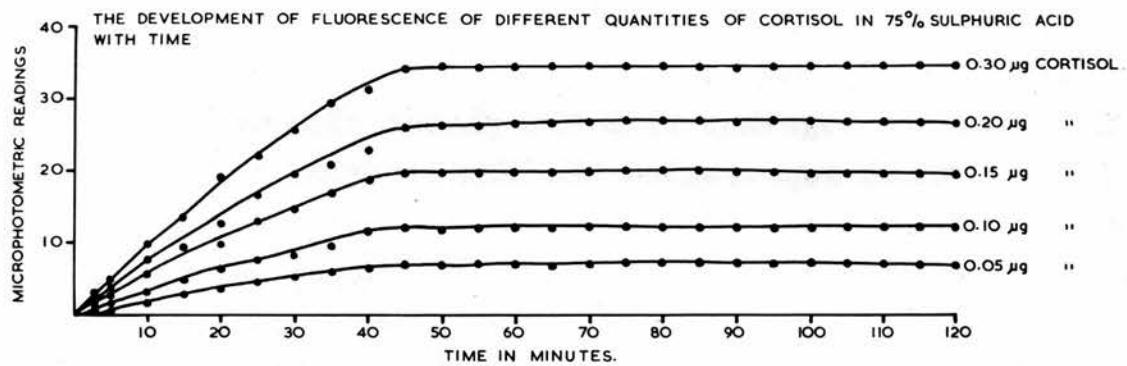


Figure (10)

that time. Similarly, the fluorescence of the plasma 2 minutes after mixing could not be attributed to either steroid as their standards did not show any fluorescence yet. To eliminate the fluorescence of the interfering substance, use was made of segment (C) of the curve which showed the rate of increase in its fluorescence with time (equal to $\tan \alpha$), together with the plasma fluorescence at 2 minutes which represented the fluorescence solely due to this substance. If it is assumed that the rate of increase in the fluorescence of the interfering substance was the same throughout its development, it would be possible to calculate its contribution to the plasma fluorescence at any time.

Two points of time were selected : the first when cortisol standard reached its maximum fluorescence (45 minutes) and the second when corticosterone standard was fully developed (80 minutes). The fluorescence of the interfering substance was calculated and was subtracted from readings of the plasma fluorescence at these two points. The remaining fluorescence was therefore due to both steroids in the plasma extract. The amount of each steroid in the plasma could be calculated from the following equations :

$$H' = F' + B' \dots\dots\dots (1)$$

$$H = F + B \dots\dots\dots (2)$$

Subtracting (2) from (1).

$$\begin{aligned} H' - H &= B' - B \\ &= xb' - xb \\ &= x(b' - b) \end{aligned}$$

$$x = \frac{H' - H}{b' - b}$$

$$y = \frac{F'}{F}$$

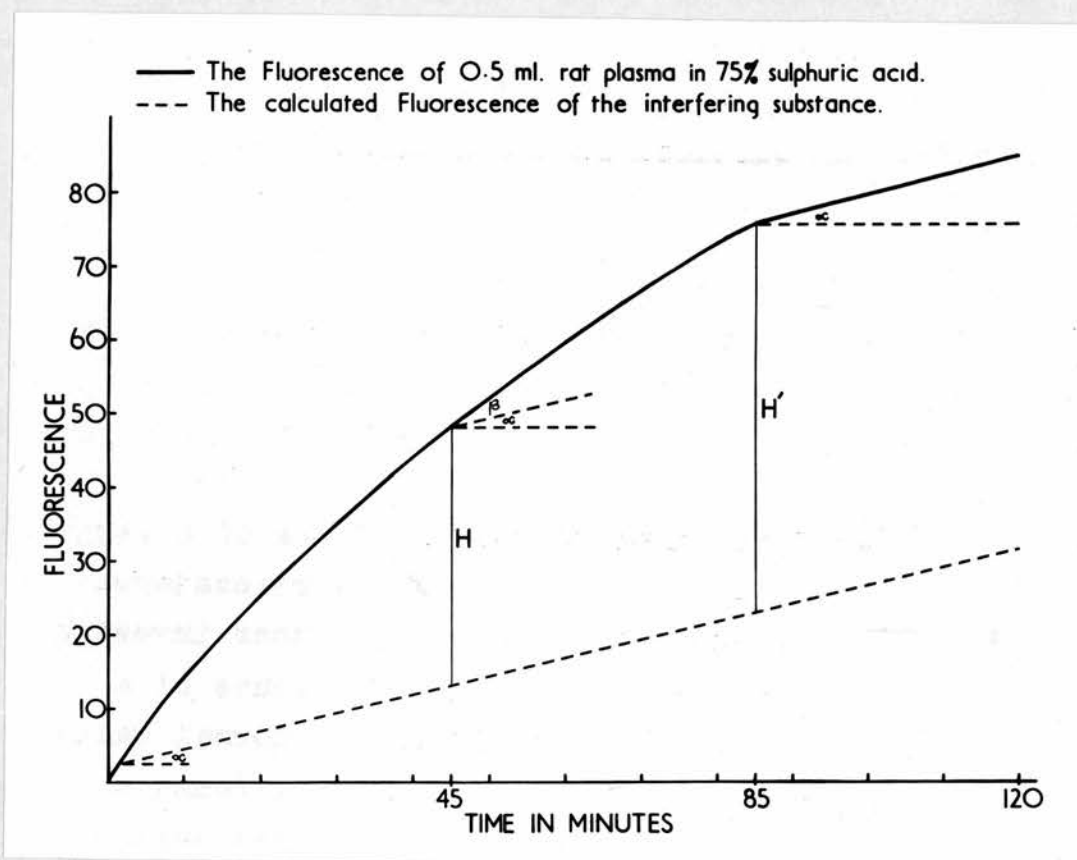


Figure (11)

where

- H', H : the fluorescence of both steroids in the plasma at 80 minutes and 45 minutes respectively.
- B', B : the fluorescence of corticosterone in the plasma at 80 minutes and 45 minutes respectively.
- F', F : the fluorescence of cortisol in the plasma at 80 minutes and 45 minutes respectively.
- b', b : the fluorescence of 1 microgram corticosterone at 80 and 45 minutes respectively.
- f', f : the fluorescence of 1 microgram
- x : microgram corticosterone in the plasma.
- y : microgram cortisol in the plasma.

DISCUSSION.

A simple method based on the fluorescence of corticosterone in sulphuric acid was devised by Silber and co-workers () to measure levels of free corticosterone in small volumes of rat plasma. In this method, attention was drawn for the first time to the existence of a residual fluorescence assayed as 4 - 8 micrograms corticosterone in 100 ml. plasma of adrenalectomized rats. Other investigators (51, 81) have all confirmed the presence of a background fluorescence of about 33% of the normal value for rat plasma. De Moor et al (33) similarly found a residual fluorescence equivalent to 3 microgram cortisol / 100 ml. human plasma. In the present work, the existence of a fluorogenic material other than cortisol and corticosterone has been confirmed in human and in sheep's plasma.

The nature of this background fluorescence has not yet been defined. It has been suggested to be probably related to cholesterol (74). However, under the conditions

of the reaction and measurement used, the fluorescence of cholesterol was only 1% that of corticosterone (Table 15).

Several attempts have been made to eliminate or make a correction for this interfering material. Silber et al (120) have proposed a correction which could be obtained by using 2 different concentrations of sulphuric acid. They found that identical aliquots of methylene chloride extract of plasma of adrenalectomized rats in 30 N sulphuric acid gave a reading of 5 - 6% that of the extract in 36 N sulphuric acid, whereas reading of corticosterone standard in 36 N was negligible. Therefore, they suggested subtraction of 5 - 6% of the higher reading from the lower reading to obtain a corrected value of zero microgram corticosterone per 100 ml. for the plasma of adrenalectomized rats.

Moncloa et al (81) tried a correction of this "non-steroidal fluorescence" by carrying out determination with several different aliquots of plasma. They found that the fluorescence was a linear function of the amount of the plasma used, extrapolation to zero plasma concentration intercepted the ordinate at a point that represented the height of the residual fluorescence.

De Moor et al (33) tried to keep the interference of the non-specific fluorescence at as low and as uniform a level as possible by measuring the plasma extract fluorescence in 75% sulphuric acid 5 minutes after mixing.

An investigation into the possibility of applying Silber's principle to human plasma, in which cortisol is known to be the predominant corticoid, was carried out. A study of the fluorescence of cortisol and corticosterone standards in different concentrations of sulphuric acid was performed. It was observed that in no acid concentration was

the fluorescence of cortisol negligible (Figure⁷). Therefore, though Silber's principle could be applied to the plasma of corticosterone-predominant animals, it was not suitable to apply in the cortisol-predominant human plasma to eliminate the background fluorescence.

Further study of the effect of time on the development of the fluorescence of cortisol and corticosterone standards in different concentrations of acid had shown that in 75% acid they did not show any fluorescence in the first few minutes after mixing whereas in all other concentrations higher than 75% they fluoresced instantly after adding the acid. In 75% sulphuric acid, the plasma extract fluoresced immediately after adding the acid and its fluorescence continued to increase even after both cortisol and corticosterone fluorescence became fully developed and steady. This phenomenon was utilised to eliminate the background fluorescence. The difference in the rate of development of the fluorescence of cortisol and corticosterone has also been utilised to calculate their respective amounts in the plasma extract.

All previous workers applying a non-chromatographic method for estimation of plasma corticoids have limited the fluorescence to either cortisol in man (33) or to corticosterone in rat (120) ignoring the small quantity of the other steroid present. This error might be negligible when referring the steroidal fluorescence to corticosterone, but the error becomes much exaggerated when expressing it in terms of cortisol only ignoring corticosterone which fluoresces with about 4 times the intensity of an equal quantity of cortisol in 75% sulphuric acid.

It is true that reading the fluorescence of the plasma

extract in 75% sulphuric acid 5 minutes after mixing, as suggested by De Moor et al (33) keeps the interfering fluorescence at its minimum level. But under these conditions the measured fluorescence is entirely due to the interfering substance, as cortisol and corticosterone standards do not yet develop any fluorescence.

iv.

FINAL PROCEDURE.

FINAL PROCEDURE.

- (1) Heparinised blood is withdrawn by venepuncture and plasma is separated immediately.
- (2) 1.2 ml. plasma is washed once with 5 volumes petroleum ether for defatting. Petroleum ether layer is discarded by means of a fine pipette connected to a vacuum suction pump.
- (3) To 1 ml. of the washed plasma pipetted into another tube is added 1 ml. N/20 sodium hydroxide solution and gently mixed.
- (4) The alkaline diluted plasma is then extracted twice with 6 ml. redistilled methylene chloride, each time by gentle inversion of the well-stoppered tube 30 times, avoiding vigorous shaking which results in emulsion formation.
- (5) After centrifugation, the extract is transferred into another tube.

The reagent blank is the methylene chloride extract of 1 ml. of water treated exactly as the plasma. The plasma extract and the blank are brought to dryness under reduced pressure in a water bath at 30°C.
- (6) The residue is quantitatively dissolved in 0.2 ml. twice redistilled ethanol, 1.8 ml. sulphuric acid 75% (V+V) is pipetted rapidly into the tubes and the mixture made homogeneous.
- (7) The fluorescence of the samples is measured in the cuvettes of an Aminco-Bowman spectrophotofluorometer; the activation wavelength is 475 mμ and the fluorescence wavelength 530 mμ. The fluorescence is measured exactly two minutes after mixing, at 45 minutes, at 85 minutes then

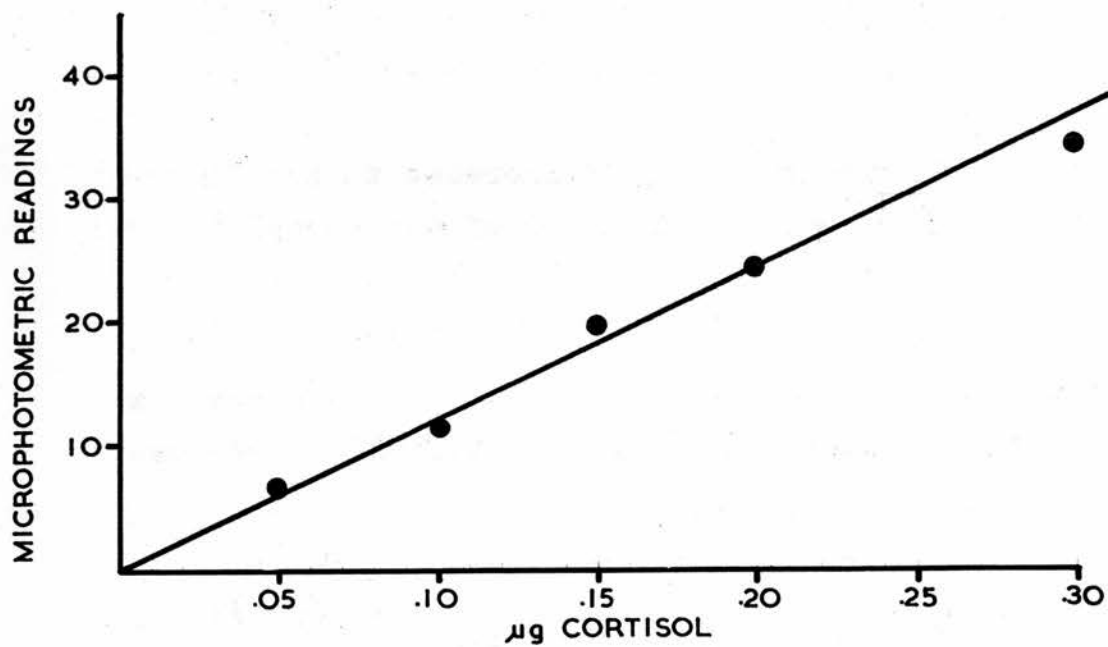
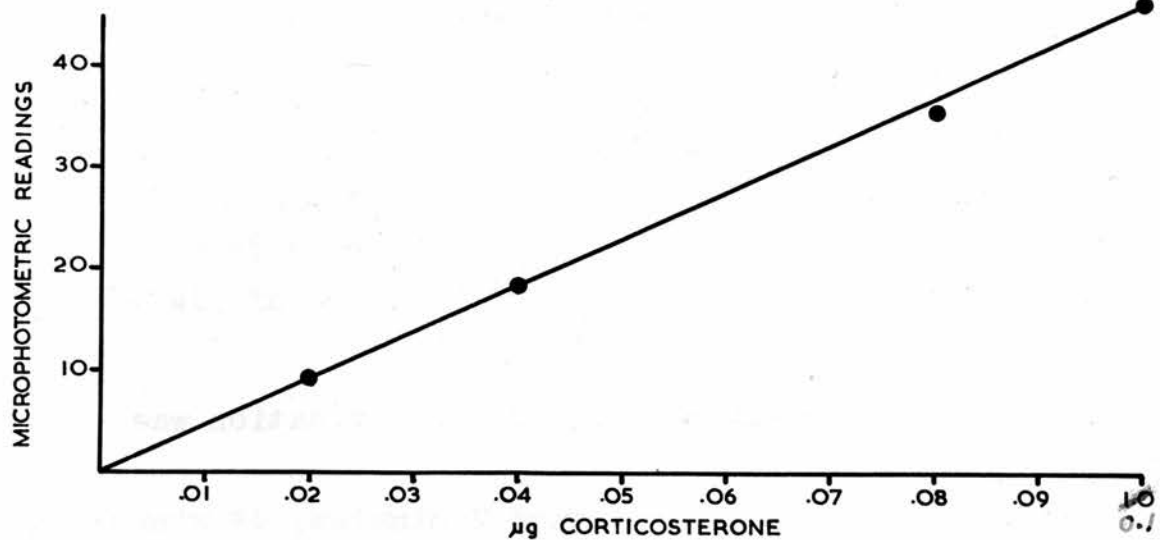


Figure (12)



every 5 minutes for 120 minutes - keeping the cuvettes in the dark, at room temperature, in between the readings

(8) A curve is plotted representing the development of the fluorescence of plasma extract during 120 minutes. The fluorescence of the interfering substance is eliminated by calculating the rate of increase in its fluorescence with time (slope of segment C of the curve) and making use of the reading at 2 minutes which represents the fluorescence solely due to this substance.

The remaining fluorescence is due to both steroids in the plasma extract ($H' & H$). The difference between the remaining fluorescence at 45 minutes and at 85 minutes ($H' - H$) is equal to the increase in the developing fluorescence of the plasma corticosterone after full development of the plasma cortisol. The amount of corticosterone in the plasma is then read directly from a curve representing the increase in the fluorescence of different quantities of corticosterone between 45 and 85 minutes. (Figure 13)

(9) The fluorescence of the plasma corticosterone, obtained from its calibration curve, is subtracted from the fluorescence due to both steroids in the plasma at 85 minutes. The remaining fluorescence is read from the calibration curve of cortisol to get the level of plasma cortisol. (Figure 12)

In this final procedure further simplification was achieved by :

(a) Reading the plasma fluorescence 2 minutes, 45 minutes and 85 minutes after adding the sulphuric acid, restricting the 5 minutes-interval readings only to segment C of the curve. This allowed the carrying out of 10 samples at a

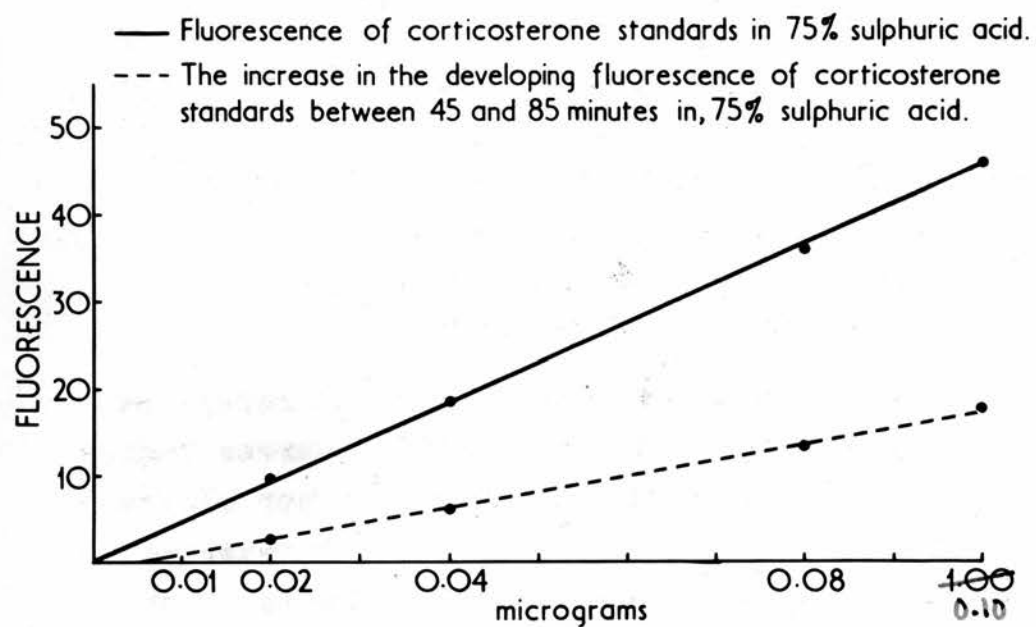


Figure (13)

time read in five successive paired groups.

(b) Omitting the routine run of the standards and calculating the results of it by reference to standard calibration curves.

In the first 30 plasma estimations, standard cortisol and corticosterone were routinely run together with the plasma samples. The readings of each standard were practically the same in the 30 experiments. Calibration curves representing the fluorescence of different quantities of cortisol ranging from 0.05 - 0.30 microgram and different quantities of corticosterone ranging from 0.02 - 0.10 microgram were plotted. Both curves were linear (Figure 12). A third curve representing the increase in the fluorescence of different quantities of corticosterone between 45 and 85 minutes was plotted against the corresponding amount. This was also linear and intercepted the abscissa at 0.005 microgram corticosterone (Figure 13).

These 3 curves were used for subsequent reference. The linear relationship in the calibration curves indicates that amounts between 0.05 and 0.3 microgram per ml. cortisol and between 0.01 and 0.1 microgram corticosterone per ml. can be accurately measured. If the amounts of the steroids in the plasma exceed this range, the plasma extract is suitably diluted before carrying out the reaction. The observation that the third curve intercepted the abscissa at 0.005 microgram corticosterone indicates that quantities below this amount per one ml. of plasma cannot be measured by this technique.

EVALUATION OF THE METHOD.

Reproducibility :

In order to determine the reproducibility of the

TABLE 19

9 replicate determinations of cortisol and corticosterone in pooled human serum.

Micrograms per 100 ml.		
	Cortisol	Corticosterone
	18.0	1.30
	20.0	1.45
	15.4	1.45
	16.3	1.70
	16.3	1.70
	20.0	1.11
	14.0	1.14
	17.6	1.14
	20.0	1.14
Average	17.51	1.40
S.D.	2.12	0.34
S.E.M.	0.71	0.11

method, a pooled human serum was washed with petroleum ether, divided into 9 aliquots 1 ml. each. The fluorometric determination for cortisol and corticosterone was carried out in all 9 samples in the same day. The results are shown in table 19. The average level for cortisol was 17.51 microgram per 100 ml. \pm 2.12 S.D. \pm 0.71 S.E.M.. The average level for corticosterone was 1.4 microgram per 100 ml. \pm 0.34 S.D. \pm 0.11 S.E.M..

Although not remarkable from the standpoint of quantitative analytical accuracy, these findings are more than adequate to demonstrate valid changes in a dynamic biological system.

Recovery : Recoveries following the addition of different amounts of cortisol and/or corticosterone are shown in table 20. Cortisol recoveries averaged 97% (range 79 - 116.5%) and corticosterone 88% (range 70-100%).

Precision : The standard error of a single determination of the free cortisol at various plasma concentrations is shown in table 21. It can be concluded that by this method, the free cortisol can be determined in concentrations generally appearing in plasma with satisfactory confidence.

For comparing the levels of cortisol and corticosterone obtained from using 0.5 ml. and 1.0 ml. of the same plasma, three different human plasma were tested, table 22.

Tentative proof of specificity of the method :

In any method there is always the question of whether one is actually measuring the compound claimed. In the steroid field, especially when fluorescence is used, this is particularly pertinent.

Of the relatively large number of corticosteroids tested

TABLE 20

Recovery of cortisol and corticosterone from serum.

microgram added		Cortisol Recovery		Corticosterone Recovery	
Cortisol	Corticosterone	µgm.	%	µg.	%
0.10	-	0.093	93	-	-
0.10	-	0.111	111	-	-
0.10	-	0.084	84	-	-
0.20	-	0.233	116.5	-	-
0.40	-	0.315	79	-	-
-	0.02	-	-	0.0172	86
-	0.02	-	-	0.0140	70
-	0.04	-	-	0.040	100
0.20	0.04	0.195	97.5	0.030	75
				Range 79-116.5%	Range 70-100%
				Average 97%	Average 83%

TABLE 21

Precision of the method for the determination of the free plasma cortisol expressed as the standard error of a single determination.

Ranges ($\mu\text{g.}\%$)	0 - 7	7 - 14	14 - 21	> 21
n	12	40	16	14
\bar{X} ($\mu\text{g.}\%$)	5.8	10.3	18.8	29.1
S(X) ($\mu\text{g.}\%$)	0.21	0.64	0.64	2.12
%	3.6	6.2	3.4	7.3

n = number of pairs of determinations.

\bar{X} = mean content of free cortisol in the plasma within the ranges.

S (X) = standard error of a single determination. This has been computed

from $S(X) = \frac{S(X' - X'')}{\sqrt{2}}$. $S(X' - X'')$ is the standard error of the difference between two independent determinations and is

$$\text{given by } S(X' - X'') = \sqrt{\frac{1}{n} \sum_{i=1}^n (X'_i - X''_i)^2}$$

% = S(X) in per cent of \bar{X}

The calculations have been done by Dr. M. Sadek, to whom I am greatly thankful.

TABLE 22

	Volume of plasma used in ml.	Cortisol $\mu\text{g.}/100\text{ml.}$	Corticosterone $\mu\text{g.}/100\text{ml.}$
Plasma I	0.5	9.40	0
	1.0	8.80	0
Plasma II	0.5	10.60	0
	1.0	10.00	0
Plasma III	0.5	7.60	0
	1.0	7.30	0

by Reichstein and Shoppee (106), Wintersteiner and Pfiffner (143), Peterson (94), Sweat (127), Goldzieher et al (48), Silber et al (120), and the steroids tested in this work, table 15, only corticosterone, cortisol, Δ^4 -pregnene - 11β , 17α , 20β , 21 - tetrol - 3 - one and Δ^4 -pregnene - 11β , 17α , 20α , 21 - tetrol - 3 - one exhibited sulphuric acid-induced fluorescence of sufficient intensity to be detected in the concentrations found in human plasma, when excited by the wavelength used in the described method and the fluorescence measured in the wavelength adopted in the procedure. The latter two compounds have not been found in human plasma and thus do not appear to present a serious obstacle to the method.

Secondly, the method described eliminates the fluorescence of any substance which behaves differently from cortisol or corticosterone with regards to the development of fluorescence with time in 75% sulphuric acid.

Recoveries from plasma averaging 97% for cortisol and 88% for corticosterone were obtained when known amounts of the steroids were added to plasma.

The final proof for the specificity of the method was obtained by carrying out several determinations by two independent workers on duplicates of the same plasma, one using the method described and the other using a paper chromatographic procedure and an isotope dilution correction and getting very similar results.

Advantages of the method :

The advantages of this simple micromethod for the simultaneous estimation of cortisol and corticosterone in 1 ml. plasma is mainly in its clinical application. It appears to be particularly useful when repeated plasma

samples are to be withdrawn. It would have a useful application in studying the diurnal variation in the level of plasma corticoids and in studying the variation of the adrenocortical function in a given individual.

The adrenal capacity has been estimated by the increase in the urinary excretion of corticosteroids after ACTH administration (135). Measurements based on the urine, however, have several disadvantages (37). A simple method for measuring plasma corticosteroids would be better suited to study adrenocortical function and its response to ACTH more directly. In the procedure outlined here, only the change in the levels of the free cortisol and corticosterone during ACTH administration would be measured. If changes in the levels of conjugated compounds due to renal impairment occurred, they would have little effect on the test.

Similarly, this method would be quite valuable for tests of acute cortisol suppression in suspected cases of suprarenal cortical hyperfunction. It has been shown, in table 15, that cortisone, prednisone, 9- α -fluorocortisol and dexamethasone, which are used as replacement therapy for hypoadrenal corticism or as a suppression test or suppression therapy for hyperadrenal corticism are non-fluorogenic, and therefore will not be measured by the method described if they are present as such in the plasma.

A particular application of the method might be in the serial study of the plasma levels of the suprarenal cortical hormones in response to surgery, an important question which is not adequately answered by urinary estimation of corticosteroid metabolite excretion. It would be

quite interesting to follow up the plasma level of the hormones after bilateral adrenalectomy.

Since plasma levels of corticosteroids are actually transport levels of these steroids and represent the equilibrium between steroid production and steroid catabolism, the rate of destruction of cortisol might be determined in clinical situations by measuring the rate of disappearance of intravenously administered cortisol. Definite alteration in this rate has been found in patients suffering from disease of the liver (19) and sometimes in patients who have undergone major surgery. (113).

The procedure described, being capable of measuring cortisol and corticosterone simultaneously in 1 ml. of plasma, lends itself to metabolic studies in small laboratory animals in addition to its clinical application in the human.

PART 11.

PLASMA CORTISOL AND CORTICOSTERONE
LEVELS IN THE NORMAL AND IN SOME
PATHOLOGICAL CONDITIONS.

INTRODUCTION.

Research in the field of the adrenal cortical function has been limited by the inability of existing techniques to measure precisely the kind and amount of the steroid elaborated by the gland. Particularly in man, the parameters have been indirect and inadequate. These have consisted principally of the alteration in certain non-steroidal constituents of the blood secondary to hormonal changes, alteration in the total number of circulating lymphocytes and eosinophils and variation in the end-products of steroid metabolism in the urine. The frequent discrepancies between the clinical picture and these indirect tests indicate that they are not always reliable indices of adrenal function.

The quantitative determination of corticosteroids in peripheral blood, on the other hand, gives a more direct picture of adrenal function. The plasma level of the hormones, ^{is} however, determined by the dynamic equilibrium existing between the secretion of the hormones and their elimination from the blood.

Several factors are concerned in the regulation of the circulating adrenal cortical steroid levels. On the positive side, the secretion of the adrenal cortex is apparently of primary importance. This depends, at least in part, on the rate at which ACTH reaches the gland. There is, however, a maximal response which cannot be exceeded by increasing the concentration of ACTH. This level probably is determined by the maximum rate of sustained secretion by

the adrenal gland (130).

On the negative side, the corticosteroids are eliminated from the blood by many factors :

1. Binding or adsorption to tissue proteins (110, 112, 114).
It is not yet possible to measure precisely the amounts of adrenal steroids in tissues, their rate of exchange or the speed of their destruction.
2. Conversion in the liver into conjugated metabolites (80).
3. Excretion through the bile (80, 114).
4. Renal clearances of the conjugated metabolites and the free hormones; the latter showing a much smaller clearance than the former. (16).

Cortisol and corticosterone are differently affected by these factors. Corticosterone appears to be more readily metabolised and conjugated (35); the concentration of conjugated corticosterone metabolites in the blood is higher than that of the corresponding cortisol metabolites (87). The enterohepatic circulation is at a minimum with cortisol but is considerable with corticosterone (114). On the other hand, the corticosterone conjugates are more slowly excreted through the kidney than those of cortisol (80).

The major component in the peripheral blood of man appears to be cortisol. Since this seems to be the principal steroid secreted by the normal human adrenal gland, the method described appears a satisfactory direct measure of adrenal cortical activity.

The second part of this thesis is concerned with the study of the concentration of cortisol and corticosterone in the peripheral venous blood of normal adults, the range of normal values and the application of the method of assay to

The distribution of plasma cortisol
in 27 normal subjects

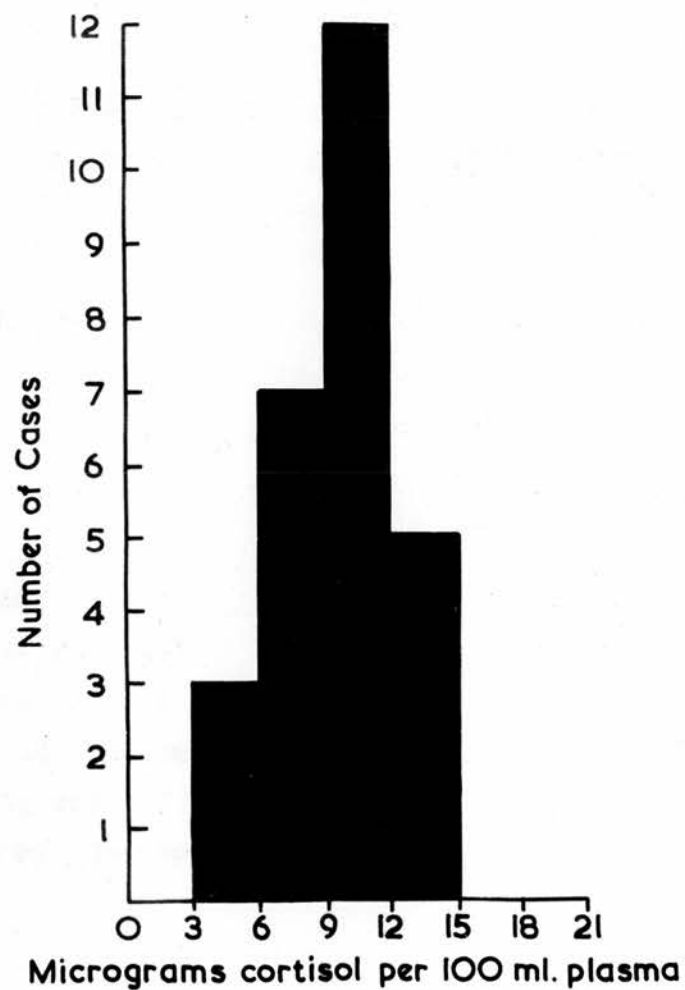


Figure (14)

some physiological and pathological conditions in which the level of plasma corticosteroids are known to be altered.

NORMAL VALUES.

Duplicate determinations were made on 27 normal adults, 15 of whom were males and 12 females.

Normal Values :

Cortisol : average 9.61 ± 2.71 S.D. Range 5-15 ug/100 ml.

Corticosterone : average 0.40 ± 0.21 S.D. Range 0-2 ug/100 ml.

Distribution of cortisol in the normal subjects (Figure 14).

3 - 6 ug/100 ml. in 11 %.
 6 - 9 ug/100 ml. in 26 %.
 9 -12 ug/100 ml. in 45 %.
 12 -15 ug/100 ml. in 18 %.

Corticosterone was found in only 25% of the cases. The mean value for plasma cortisol in the females was 10.1 ug/100 ml. compared to 9.2 ug/100 ml. in the males. A sex difference, if any exists, must be small. No statistically significant difference was noted in the plasma cortisol level in the various age groups tested, between 15 and 65 years.

DISCUSSION.

Cortisol values obtained in this study conform to values reported by other laboratories (16, 37, 121, 129) and establish the clinical usefulness of the technique.

TABLE 23

Values of plasma cortisol and corticosterone in 27 normal adults

Name	Sex	Age	μ g. per 100 ml.	
			Cortisol	Corticosterone
N.P.	F	22	8.4	0
M.H.	F	22	12.4	0
A.C.	F	22	9.4	0
A.S.	F	24	9.2	0
J.F.	F	25	6.2	0
M.M.	F	28	8.0	0
E.S.	F	30	10.0	0
A.J.	F	45	15.0	1.35
M.S.	F	45	9.2	0
J.S.	F	48	14.6	0
P.S.	F	55	5.6	1.55
E.S.	F	63	13.8	0
R.M.	M	18	10.4	0
D.K.	M	19	9.6	0
J.B.	M	24	12.0	0
H.M.	M	25	9.2	0
T.I.	M	26	10.8	1.6
M.S.	M	30	6.2	0
A.S.	M	30	8.4	0
M.S.	M	31	10.8	0
F.R.	M	35	10.0	0
G.S.	M	40	7.4	0
J.M.	M	45	8.4	1.35
G.B.	M	49	5.0	2.00
R.M.	M	50	13.0	0
A.W.	M	50	5.0	1.55
A.A.	M	50	11.6	1.35

N.B. Heparinised blood was withdrawn between 9.00 and 10.00 a.m.

TABLE 24

Comparison of Plasma Cortisol and Corticosterone levels found by various investigations using various methods.

Author	Method	Cortisol μg./100ml.	Corticosterone μg./100ml.
Morris & Williams 1953	Partition chromatography then polarography of Girard hydrazones	6.5 - 10.5	4 - 10.5
Morris & Williams 1955	Partition chromatography then U.V. absorption and BTZ.	7.8 - 15.8	7.8 - 15.8
Weischelbaum & Margraph 1955	Column chromatography then BTZ minus Porter-Silber	9.5	(17-deoxysteroids) 8.9
Sweat 1955	Silica gel chromatography then sulphuric acid fluor.	10.8	4.3
Takeda 1956	Silica gel chromatography then phosphoric acid fluoresc.	(cpd. F-like subs) 8.6	(cpd. B-like subs) 8.4
Ely et al 1958	Florisil chromatography then silica gel chromato- graphy and sulphuric acid fluoresc.	10.9	3.0
Starlinger & Tamm 1955	Modified Sweat's method	9.6	1.6
McLaughlin et al 1958	Modified Sweat's method	12.4	2.0
Lewis 1957	Paper chromatography then sulphuric acid fluorescence	9.2	1.2
Bush & Sandberg 1953	Silica gel prechromatography then paper chromatography	10-12	None to 2
Peterson 1957	Paper chromatography then sulphuric acid fluorescence with isotope dilution for recovery correction	6.0-21	0.5-2.0
Present Study	Sulphuric acid fluorescence of the methylene chloride plasma extract	9.61	None to 2

There is a considerable disagreement between the different workers on the absolute quantity of corticosterone in human peripheral blood and its relative ratio to cortisol. Some workers have reported values for plasma corticosterone as high as those for cortisol and even higher (84,141). Others have obtained results which are completely opposed to these, in which the plasma concentration of corticosterone was very low, often absent altogether and the cortisol level was more than six times that of corticosterone (27,94). Table 24 compares the plasma levels of both steroids found by various investigators using various methods.

The reported high values of corticosterone by some workers could either reflect an actually high plasma level of this hormone or could be accounted for by the presence of substances interfering with the method of determination and not completely separated from the steroid.

In Sweat's method (129), the fraction eluted as corticosterone is always contaminated by other fluorogenic material (Sweat cited by 26). There is also tailing and overlap of the steroid zones. Takeda (132), using paper chromatography of the corticosterone elution fraction obtained from silica gel column chromatography of human plasma, found two substances other than corticosterone. Ely et al (38), tried to improve on the purification step in Sweat's original method by substituting Florisol column chromatography for the pentane-alcohol partition step. By this modification, the mean value for corticosterone was reduced from 4.3 to 3.0 ug per 100 ml. but they still suspected that the modified technique was not wholly specific for corticosterone.

The corticosterone eluted fraction in Morris and Williams methods was identified on the basis of its BTZ reducing property, U.V. absorption at 240 mμ and its indistinguishable polarographic wave from corticosterone standard. Morris and Williams themselves (85) have demonstrated the presence of large amounts of non-specific substances in the crude plasma extract which give both the BTZ reaction and the U.V. absorption peak. Butt and Crooke (28) have similarly demonstrated the presence of material in the unfractionated plasma extract which gives polarographic wave indistinguishable from that given by some corticosteroids in the method using the Girard hydrazones. A possible explanation for such high corticosterone values in Morris and Williams methods is the presence of some interfering substance in spite of their extensive separation and purification.

The reported low values for corticosterone might be due either to a really low plasma value or to an inefficient extraction or loss during purification and separation. However, Bush and Sandberg (27), in spite of using a large volume of plasma (175 - 300 ml.) found very low levels of corticosterone. Recovery of different amounts of corticosterone from plasma was as good as that of cortisol in all previous reports giving low corticosterone values as well as in this present study. The isotope dilution technique used by Peterson (94) for recovery correction is strongly against the possibility of corticosterone loss as an explanation of its low plasma level. This discussion tends to support the view that the human adrenal, under normal conditions, secretes mainly cortisol. The fact that the urine contains mainly metabolites of cortisol and very little corticosterone metabolites (26) is a further evidence for

TABLE 25

The method was applied for the estimation of cortisol and corticosterone in 5 sheep's sera and 3 rat's plasma

Sheep μg./100ml.		Rat μg./100ml.	
F	B	F	B
9.00	0.80	0	18.8
10.70	0	1	10.0
12.60	0	0	16.2
4.00	0		
9.10	0		

this view, particularly as it has been demonstrated that such metabolites are excreted after corticosterone administration to human beings (108).

Cortisol and corticosterone in sheep's serum and in rat's plasma :

The method was applied for the estimation of cortisol and corticosterone in 5 sheep's sera and 3 rat's plasma, table 25. Cortisol in sheep and corticosterone in rats were found to be the predominant steroids as previously reported by other workers (23).

Plasma Cortisol and Corticosterone in Pregnancy :

In nine cases of apparently normal pregnancy at 36 weeks, the plasma cortisol ranged from 18.1 - 27.5 microgram /100 ml., average 24.4 ± 3 S.D., and corticosterone ranged from 0 - 2 microgram /100 ml., average 0.67 ± 0.72 table 26.

Similar high plasma cortisol values have been reported by various workers (7, 44, 79, 83, 140). Such high levels of the free cortisol do not appear to be due to increased corticoid production but seem to be mainly related to a disturbance in cortisol catabolism characterised by a slower rate of reduction and/or conjugation of this steroid (79).

Plasma Cortisol and Corticosterone in some patients with hypo- and hyper adrenal corticism :

Plasma determinations were performed in one patient with Addison's disease, three patients with hypopituitarism and secondary adrenal insufficiency, one patient with clinically manifest Cushing's syndrome and two patients with hirsutism in whom suprarenal cortical hyperfunction was

TABLE 26

Plasma Cortisol & Corticosterone in 36 weeks
normal pregnancy.

Name	$\mu\text{g.}/100\text{ml.}$	
	Cortisol	Corticosterone
M.K.	27.5	2.0
S.K.	18.1	1.5
W.N.	25.4	-
L.A.	23.0	0.66
R.S.	22.6	-
O.N.	24.4	-
N.R.	24.4	0.5
C.N.	21.8	-
F.N.	19	1.35
Average	24 ± 3	0.67 ± 0.72

Plasma Cortisol in normal and various physiological and pathological conditions

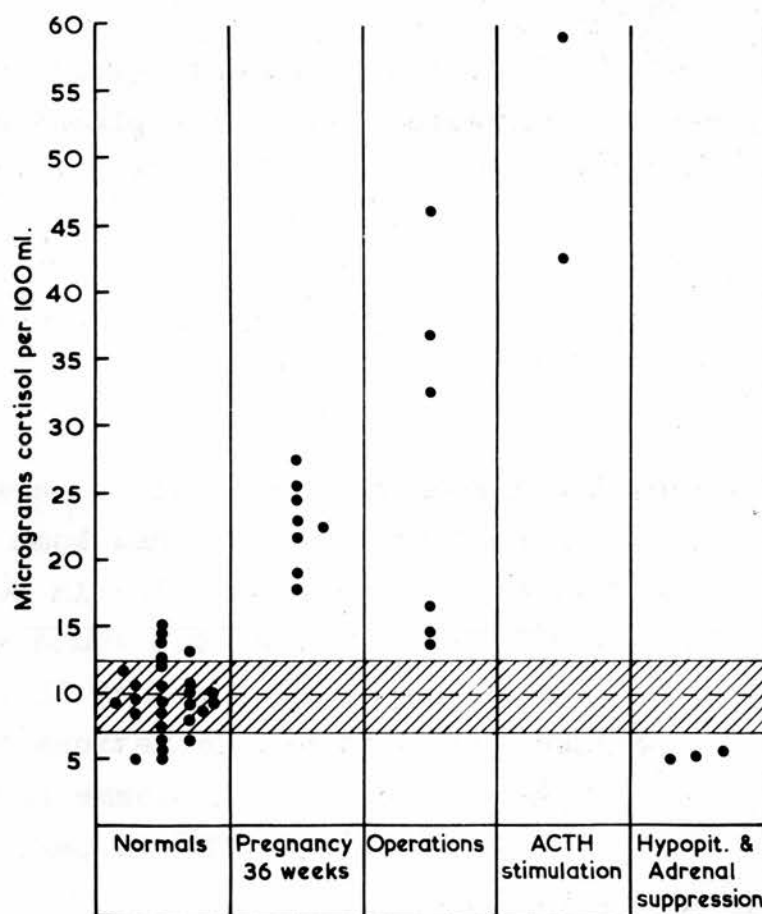


Figure (15)

suspected, table 27 and figure 15.

In the patient with Addison's disease and in one case of hypopituitarism the plasma cortisol was within the normal limits. In the two other cases of hypopituitarism the plasma cortisol was below normal. Only in the clinically manifest case of Cushing's syndrome was it definitely elevated. Acute cortisol suppression by 1 mgm. fluorohydrocortisone daily for 3 days resulted in lowering the plasma level to 5 ug/100 ml.. This patient was proved to have bilateral adrenal hyperplasia on subsequent operation. In the other two suspected cases, the plasma cortisol level was within the normal limits but the adrenal response to ACTH (60 I.U. corticotrophin gel 12-hourly for 2 days given I.M.) resulted in an increase in plasma cortisol level more than five-fold, suggesting suprarenal cortical hyperfunction, figure 16

In patients with both primary and secondary adrenocortical hypofunction, the plasma cortisol values were either low or within the normal range. This has been the experience of other investigators (37,140). In both groups the use of an ACTH-plasma response test would be a more informative index of adrenal insufficiency than any number of single plasma corticoid determinations (77). In suprarenal cortical hyperfunction a high plasma cortisol level and/or an exaggerated response to ACTH are particularly useful in confirming the diagnosis.

The effect of surgery on the plasma levels of cortisol and corticosterone :

The plasma levels of cortisol and corticosterone were determined in six non-endocrine patients undergoing operat-

Plasma cortisol in response to ACTH stimulation

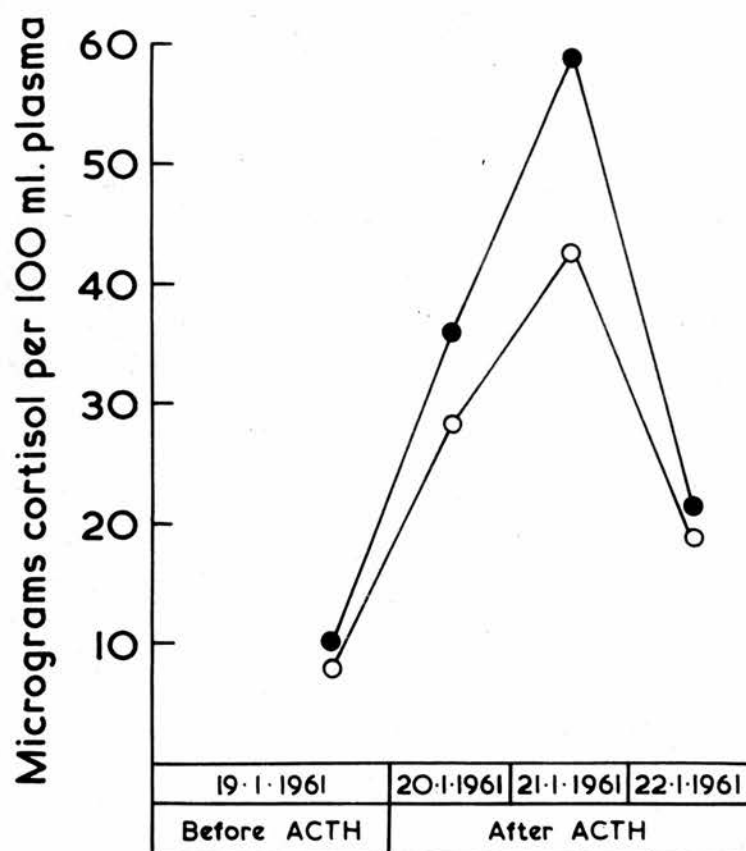


Figure (16)

TABLE 27

Plasma Cortisol and Corticosterone in some patients with hypo- and hyperadrenal corticism.

Patient	Sex	Age	Diagnosis	Medication	μ g. per 100 ml.	
					Cortisol	Corticosterone
M		31	Addison's	Cortisone	9.6	-
F		54	Hypopituitarism	Cortisone & thyroxin.	5.4	-
F		47	Hypopituitarism	Cortisone & thyroxin	10.4	-
F		54	Hypopituitarism	None	5.8	-
M		35	Cushing's	None		-
M		35	Cushing's	9 α -Fluoro hydrocortisone	5.0	-
F		34	? Cushing's	None	10.0	-
				A.C.T.H.	59.0	1.5
F		34	? Cushing's	None	8.0	-
				A.C.T.H.	43.0	-

TABLE 28

Plasma Cortisol (F) and Corticosterone (B) in response to surgical operations (ug./100ml.)

Name	H.Y.	G.S.	D.G.	F.N.	A.S.	K.L.
Age	46	50	35	30	28	30
Sex	F	F	F	F	M	F
Operation	Hysterectomy	Excision of Vaginal Sarcoma	Removal of a polyp.	Dilation & Curettage	Correction of Colle's Fracture	Dilatation & Curettage
CS.	F	B	F	F	F	B
Pre-op	16	1.35	9.6	1.05	18.4	1.35
Operation	46	-	36.8	-	32.3	-
2nd. day	17.8	-	19.6	-	17.2	-
3rd. day	9.2	-	5.8	-	12.4	1.5
4th. day	4.6	1.50			14.6	
5th. day	5.6	1.50			14.6	
6th. day	8.0	1.60			14.6	
					8.4	13.8
					16.4	13.8
					16.4	-

ions of various degrees of severity. A basal determination was performed on the pre-operative day then blood samples were taken immediately after the operation and at 9.00 a.m. each morning for the next few post-operative days. Results are shown in table²⁸ and figure ¹⁷.

Three patients had a slightly elevated concentration of the plasma cortisol on the pre-operative day. Following surgery, an increase in the plasma level of cortisol was noted in four patients; this increase was proportional to the duration and severity of the operative procedure. In one of the patients followed up for 6 days, a drop in the free plasma cortisol to a subnormal level was observed on the fourth post-operative day, followed by a gradual rise to the normal level.

Corticosterone was detected pre-operatively in the plasma of three patients. It then disappeared on the operation day when plasma cortisol attained its highest level. It reappeared in the plasma in three patients afterward with the decline of cortisol.

Discussion : the pre-operative plasma 17-hydroxycorticosteroid concentrations were reported by several observers to be slightly higher than normal (42,134). This might indicate that the underlying disease and the psychological tension of the approaching operation were causing a slight activation of the pituitary-adrenal system. However, this pre-operative rise was not a constant phenomenon.

It has been well established that surgical trauma is accompanied by an immediate and marked rise in the level of the free corticosteroids in the plasma of most patients (46,65,113, 134). This post-operative rise has been

Plasma cortisol in 3 patients in response to operation.

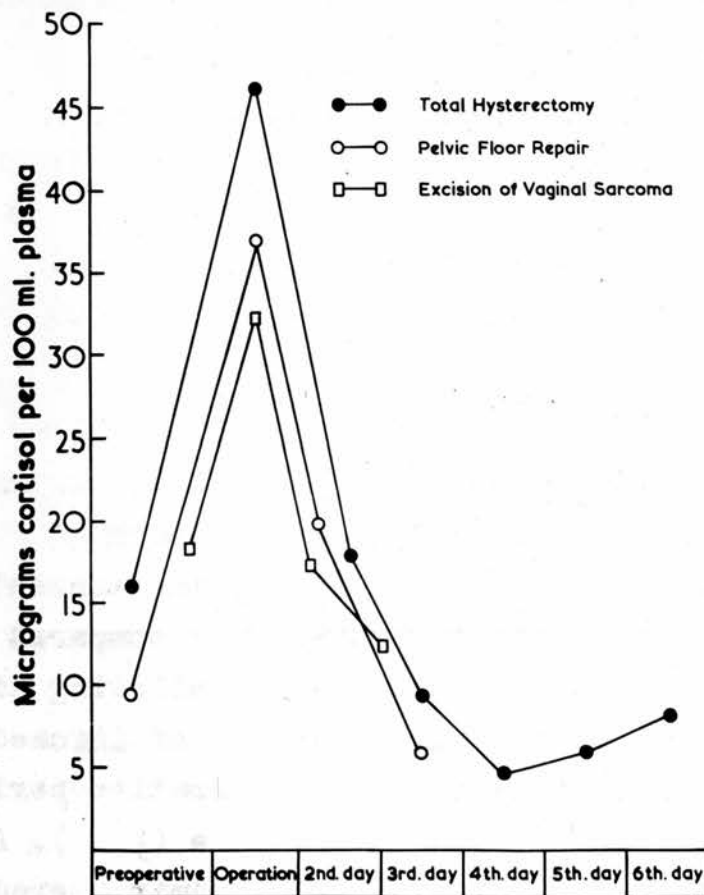


Figure (17)

correlated with the length of the operation and the extent of the tissue trauma (113). The plasma level might double within an hour of the beginning of the operation, reach a maximum within 10 hours then gradually return to normal within 3 - 5 days, decreasing rather quickly in the first post-operative day and afterwards more slowly (134). After the second or third post-operative day there sometimes appeared a short period of sub-normal 17-hydroxycorticosteroids in the plasma followed by a secondary smaller rise (46).

The rise in plasma cortisol is partly due to increase production of the endogenous hormone in response to the stresses of anaesthetic and surgical trauma (34). However, factors other than adrenal cortical stimulation play a part in raising the steroid concentration during and subsequent to surgery.

The rise in the level of free 17-hydroxycorticosteroids following operation was greater than the rise in normal subjects following maximal stimulation with ACTH (126). On the other hand, the rate of increase in the concentration of conjugates was slower post-operatively as compared to their rate of increase in normal subjects following administration of ACTH (126). Impaired removal of infused cortisol was observed in the immediate post-operative period in patients undergoing major surgical procedure (86). Also, when identical infusions of cortisol were administered to a series of adrenalectomised dogs subjected to immobilisation, anaesthesia and surgery, a significantly higher plasma level of free 17-hydroxycorticosteroids occurred in the anaesthetised animals than was found in the conscious immobilised ones (125).

All these evidences point to a delay in the conjugation

of free plasma cortisol following operation. It seems well accepted nowadays that the increased plasma free cortisol levels after surgery are the result of both increased secretion by the adrenals and impaired hepatic removal from the circulation.

There are few reports on the effect of surgery on the plasma level of corticosterone. In this study, free corticosterone disappeared from the plasma in response to operative intervention at least for 24 hours. This might well be related to the fact that corticosterone is more rapidly metabolised than cortisol (80), being thus, probably, used up by the stress tissues before the more active metabolic hormone cortisol is called upon to meet the metabolic effects of trauma.

SUMMARY.

SUMMARY.

1. The literature of the various methods of estimation of the free plasma corticosteroids has been reviewed, and the fluorometric method of assay has been preferred on account of its simplicity and marked sensitivity.
2. A simplified procedure for the estimation of free cortisol and free corticosterone in human plasma has been described. It involves methylene chloride extraction and direct measurement of the fluorescence in 75% sulphuric acid without purification or separation of the steroids. This technique was shown to be precise, simple, very sensitive and reasonably specific and to yield good recoveries of the added cortisol and corticosterone to the plasma. In spite of certain specificity limitation, it has practical applications in estimating these two steroids in small samples of plasma volumes (1 ml.) and in studies which are designated principally to demonstrate increases or decreases in the adrenocortical hormones without absolute specificity.
3. A procedure has been described which can be applied to human plasma as well as to rat or rabbit plasma to correct for the interfering fluorescent material in the unfractionated plasma extract. This procedure depends on the comparison between the rate of development of the fluorescence of the plasma and of standard solution of cortisol and corticosterone. The difference in the rate of development of cortisol and corticosterone fluorescence has been utilised to calculate their respective amounts in the plasma extract.
4. The mean value for free plasma cortisol in 27 normal

adults was 9.61 ± 2.71 and for free plasma corticosterone 0.40 ± 0.21 microgram per 100 ml. In 9 cases of normal pregnancy at 36 weeks, the average free plasma cortisol was 24 and corticosterone 0.67 micrograms per 100 ml. A rise in the free cortisol and a disappearance of free corticosterone from the plasma was observed in response to surgery.

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